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JOURNAL OF ANATOMY

SPINAL ORIGIN OF THE VENTRAL SUPRAOPTIC DECUSSATION (GUDDEN'S COMMISSURE) IN THE SPIDER MONKEY*

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INTRODUCTION

The origin of the fibres in the ventral supraoptic decussation has been disputed since its discovery by von Gudden (1870). Earlier workers were largely concerned with demonstrating Gudden's commissure as an anatomical entity. Attempts to determine the source and destination of its constituent fibres have been inconclusive, although various structures above the midbrain, especially the medial geniculate body, have been suggested as sites of origin and termination. That a component of Gudden's commissure originates in the spinal cord has been completely unsuspected, apart from a suggestion by Magoun & Ranson (1942) that such fibres might come from levels below the pons.

In studying the ascending fibre systems from the caudal segments of the spinal cord in the spider monkey, secondary degeneration was seen in Gudden's commissure. At first, considered as an artefact, it was subsequently verified in six preparations. The degenerated fibres in Gudden's commissure, together with other degenerations in some of those specimens, were illustrated graphically in one of our earlier communications to this *Journal* (Chang & Ruch, 1947). The existence of this system of fibres alters our conception of the functional significance of Gudden's commissure, which is based on the belief that it serves as an interconnection between the medial geniculate bodies or inferior colliculi of the two sides.

MATERIAL AND METHODS

Lesions were made aseptically at selected levels of the spinal cord in five spider monkeys (*Ateles*) and one cat. A second operation was performed on one animal in which the caudal segment of the spinal cord had been divided 6 months previously.

Various periods of time (from 14 to 30 days) were allowed for secondary degeneration of the ascending nerve fibres. Under deep Nembutal anaesthesia, the vascular system was perfused with a mixture of equal parts of 3% magnesium sulphate and 3% potassium dichromate, without previous washing with saline. The brain and spinal cord were removed immediately, and placed in 10% neutral formalin. Every precaution was taken to avoid unnecessary handling of the nervous tissue. After 24 hr. the brain and critical segments of the spinal cord were sliced into sections.

* This paper constitutes a part of a dissertation presented to the Faculty of the Graduate School of Yale University, by H. T. Chang in candidacy for the Degree of Doctor of Philosophy. Aided by a grant from the Fluid Research Fund, Yale University School of Medicine.

about 3 mm thick, and trimmed. Selected slices were then transferred, without washing, to the chlorate-osmic-formalin mixture of Swank & Davenport (1935).

The brain slices were placed flat on a sheet of blotting-paper in a Stender dish which was closely covered and left in the dark. The mixture was agitated, and the pieces of tissue turned over daily. After 8 days the slices were washed, dehydrated with alcohols and embedded in celloidin in the shortest possible time. Satisfactory results were obtained by completing the whole process from dehydration to mounting in 2 days. Sections were cut 50μ thick, and mounted in balsam with glass cover slips.

RESULTS

Experiment 1 Spider monkey series no 13, adult male Spinal transection at Ca₁ (1 Sept 1944) Killed 1 Oct 1944

Lesion At the level of the lesion, the nervous tissue was completely destroyed, and replaced by scar tissue. Microscopical examination reveals no intact nerve fibres there.

Histological examination Immediately above the lesion degenerated fibres cross in the ventral commissure, pass through the grey matter of the ventral horn, and reach the ventrolateral funiculus. A compact group of degenerated fibres, which can be traced throughout the spinal cord and medulla, is present in the periphery of Gowers' ventrolateral fasciculus. In the midbrain, a number of the degenerated fibres are grouped in the dorsal part of the posterior commissure. The rest of them curve ventrolaterally through, or over, the ventral part of the medial geniculate body and join the fibre bundles along the medial border of the optic tract. Fibres also pass through the dorsal aspect of the basis pedunculi. At the anterior end of the thalamus, the course of the degenerated fibres can be followed along the dorso-medial border of both optic tracts (Pl 1, fig 1). Those fibres do not intermingle with the fibre bundles of the dorsal supraoptic decussation—*pars ventralis* (Meynert's commissure)—although they are in close relation to it. On the other hand, the decussating degenerated fibres are conspicuously present in Gudden's commissure. After crossing they travel along the border of the optic tract toward the ventromedial part of the globus pallidus, but none of the fibres actually enters that nucleus. The final destination of these fibres is not determinable, because the serial sections at this level are incomplete.

Experiment 2 Spider monkey series no 17, one-year-old female Spinal transection at Ca₁ (28 Nov 1944) Killed 11 Dec 1944

Lesion Sections at the level of the transection showed that the spinal cord was completely interrupted. Damage to the spinal cord was somewhat greater on the right side than on the left.

Histological examination Degenerated fibres on both sides of the cord can be traced from Gowers' fasciculus to the midbrain and thalamus. In this specimen, the midbrain and thalamus were cut in a more frontal plane, giving sections different in appearance from those in Experiment 1. Sections cut at the level of the posterior commissure show a few degenerated fibres, emanating from the spinothalamic tract in the dorsolateral tegmental region, which turn laterally and then ventrally through

the medial geniculate body to its lateral border. Traced upward through several sections, these fibres can be followed to the medial border of the optic tract near the lateral geniculate body. Relatively more abundant are the degeneration products in the fibre layer between the medial geniculate body and the lateral geniculate body. Degenerated fibres are seen in the dorsolateral aspect of the basis pedunculi where their orientation suggests their coming from the region of the nucleus peripeduncularis and the posteroventral border of the thalamus. In some sections, especially on the right side, degeneration products are seen about the medial region of the nucleus pregeniculatus where they seem to terminate (Pl 1, fig 2). In other sections, fibres near the nucleus pregeniculatus shift dorsally towards the ventral nucleus of the thalamus. At still higher levels, dark degeneration granules are arranged in rows along the junctional border of the optic tract and the fibre bundle of Meynert's commissure which is distinguished by its lighter colour in the Marchi preparations. Degeneration in the optic chiasma is confined to Gudden's commissure.

Experiment 3 Spider monkey series no 14, adult female Left hemisection at Ca₁ (8 Sept 1944) Killed 26 Sept 1944

Lesion The hemisection of the cord was complete except for a part of the funiculus sulcomarginalis near the ventral median sulcus. On the right, only the medial margin of the dorsal column was involved.

Histological examination Ascending degenerated fibres are present on both sides in the fasciculus ventrolateralis superficialis of Gowers, but are more numerous on the operated side throughout the spinal cord. Before reaching the thalamus, some fibres leave the dorsal tegmental region of the midbrain to curve dorsally or ventrally or to pierce the anterior part of the medial geniculate body, and finally join the most medial fascicle of the optic tract. Sections from the anterior mesencephalon clearly show that degenerated fibres sweep over the most dorsal aspect of the pes pedunculi, and go to the medial border of the optic tract where they enter the lateral geniculate body. Many various-sized particles of degeneration material are collected in the tricuspid area formed by the pes pedunculi, medial geniculate body and the lateral geniculate body (Pl 1, fig 3). This can be seen on both sides of the brain.

Pl 1, fig 4 demonstrates the presence of degenerated fibres in the posterior part of the optic chiasma, where the unaffected dorsal supraoptic decussation can be seen lying between the third ventricle and the degenerated fibres.

Experiment 4 Spider monkey series no 18, adult female Unilateral anterolateral chordotomy at C₃ on the right (5 Jan 1945) Killed 26 Jan 1945

Lesion Although the primary lesion was confined to the anterolateral funiculus of the right side, some fibres in Flechsig's fasciculus also underwent degeneration, which was probably the result of an accidental trauma or of a vascular disorder occasioned by the near-by lesion. But this does not affect the degeneration picture in the midbrain and thalamus. The primary lesion was a triangular area with an obtuse angle directed medially. The side along the ventrolateral periphery was about 2 mm in length. The dorsal spinocerebellar tract and the pyramidal tract escaped direct involvement in the lesion. The other side of the cord was normal.

Histological examination In the anterior part of the midbrain degenerated fibres

are seen in the medial lemniscus, and occupy a fairly large area in the dorsolateral tegmentum on the operated side. The corresponding area on the other side is free of degeneration. At the level of the posterior commissure, the degenerated fibres begin to radiate in various directions. Some, which are directed ventrolaterally, pass through the medial geniculate body and the dorsolateral border of the pes pedunculi. Some degeneration product is deposited in a tricuspid area formed by the nucleus peripeduncularis, and the medial and lateral geniculate bodies. On the other side of the same section, the medial geniculate body contains no degenerated fibres. From the dorsolateral aspect of the pes pedunculi the degenerated fibres can be followed along the medial border of the optic tract to the optic chiasma where they cross in Gudden's commissure (Pl 2, fig 7). The number of degenerated fibres in Gudden's commissure in this specimen is greater than in any of the other animals studied. The distribution of the degenerated fibres in the optic tracts is symmetrical.

Experiment 5 Spider monkey series no 19, adult female First operation spinal transection at Ca_1 (13 July 1945) Second operation left hemisection at L_1 (10 Jan 1946) Killed 30 Jan 1946

Lesions Histological sections at the site of the first lesion were not made, but the degeneration picture in the segment above the lesions suggests a complete transection. The medial margin of the second lesion is sharp and does not extend to the right side. The hemisection is complete apart from a very narrow strip along the median line.

Histological examination At the level of the inferior colliculus, degenerated fibres stream dorsally parallel, and largely medial, to the lateral lemniscus. They apparently turn dorsally before the medial lemniscus fibres do, since the main body of the latter still remains in the tegmentum near the pons while the majority of the former has already reached the parabigeminal region beneath the inferior colliculus. At the junction of midbrain and thalamus some degenerated fibres leave the main stream of thalamopetal fibres, pass through the nucleus peripeduncularis around the dorsal aspect of the pes pedunculi and proceed to the medial border of the optic tract. There are few of these fibres—the fewest encountered in the five spider monkeys observed. This system of degenerated fibres, coursing within Gudden's commissure, reappears in the optic tract of the opposite side. The position of the fibres in the chiasma is ventral to Meynert's commissure. The small fibre strand of Ganser's commissure stands out beneath the recess of the third ventricle as a separate unit.

ORIGIN, COURSE AND TERMINATION OF THE SPINAL COMPONENT OF GUDDEN'S COMMISSURAL FIBRES

Origin Degeneration of fibres in the ventral supraoptic commissure was produced by transection, hemisection and anterolateral section of the spinal cord at various levels. It is evident that some of the fibres in Gudden's commissure have their cells of origin in the spinal cord.

The lower limit of the spinal origin is established by the fact that secondary degeneration was present in Gudden's commissure after transection of the spinal cord at the first caudal segment. Cells in the conus of the spinal cord must contribute

some fibres to that commissure which, therefore, carries some of the longest ascending fibres in the central nervous system

The caudal cord region is not the sole origin of the supraoptic decussational fibres in question, since lesions at higher levels of the spinal cord produce more decussational degeneration than does a spinal transection at the caudal region. Especially, the number of degenerated fibres in Gudden's commissure is greater (though not very much greater) in spider monkey no. 18 (with an anterolateral chordotomy at C_3) than in the animals no. 13 and no. 17 (spinal transection at Ca_1) or no. 14 (spinal hemisection at Ca_1). Thus, additional fibres must be contributed from the segments between C_3 and Ca_1 . Furthermore, degenerated fibres in Gudden's commissure are demonstrable following a hemisection at the level of the first lumbar segment of a monkey in which the caudal cord had been transected 6 months previously. The appearance of new degeneration in the decussation proves that the lumbar and sacral segments contribute fibres to the ventral supraoptic decussation. Therefore, it is believed that this system of fibres originates from all levels of the spinal cord, but the contribution from the caudal segments of the spider monkey, which are very highly developed (Chang & Ruch, 1947*a*), seems disproportionately great. It remains unknown which group of cells in the grey matter of the spinal cord gives origin to these fibres.

Unilaterality versus bilaterality Hemisection of the spinal cord gives rise to some degeneration in the anterolateral funiculus of both sides. However, degenerated fibres traversing the medial geniculate body are present only on the operated side (Pl 2, fig. 6). The medial geniculate body on the unoperated side is virtually free of degenerated fibres (Pl 2, fig. 5), despite their fairly large number in the anterolateral column on the unoperated side. It is assumed that the degenerated fibres ascending contralaterally to the lesion are of the same category as the bulk of the fibres ascending on the side of the lesion—namely, spinothalamic, ventral spinocerebellar fibres, etc.—originating at the site of the lesion, or crossing obliquely through the lesion. Since such contralateral fibres do not pass through the contralateral geniculate body, as mentioned above, it follows that similar fibres crossing the spinal cord below the lesion and ascending on the side of the lesion are not responsible for the degeneration in the ipsilateral medial geniculate region. This suggests that fibres ascending from the lesion to the decussation must originate on the same side of the spinal cord as, and below the level of, the lesion.

Course If the above is correct, axons destined for the supraoptic decussation, after emerging from cells in the grey matter of the spinal cord, turn laterally to the ventrolateral funiculus on the same side, where they intermingle with the fibres of other ascending systems and ascend the cord without much change in course. In the lower medulla, they occupy, along with the others, a region between the inferior olive and the descending trigeminal tract at the ventrolateral periphery of the medulla. After the divergence of the spinobulbar and spinocerebellar components from the composite fibre bundle of Gowers' fasciculus, they proceed farther upwards in company with the spinocollicular and spinothalamic fibres. They cannot be distinguished topographically from the fibres of the other systems throughout their ascending course until the posterior end of the thalamus is reached, where they curve laterally and pass through the dorsal end of the basis pedunculi, the medial

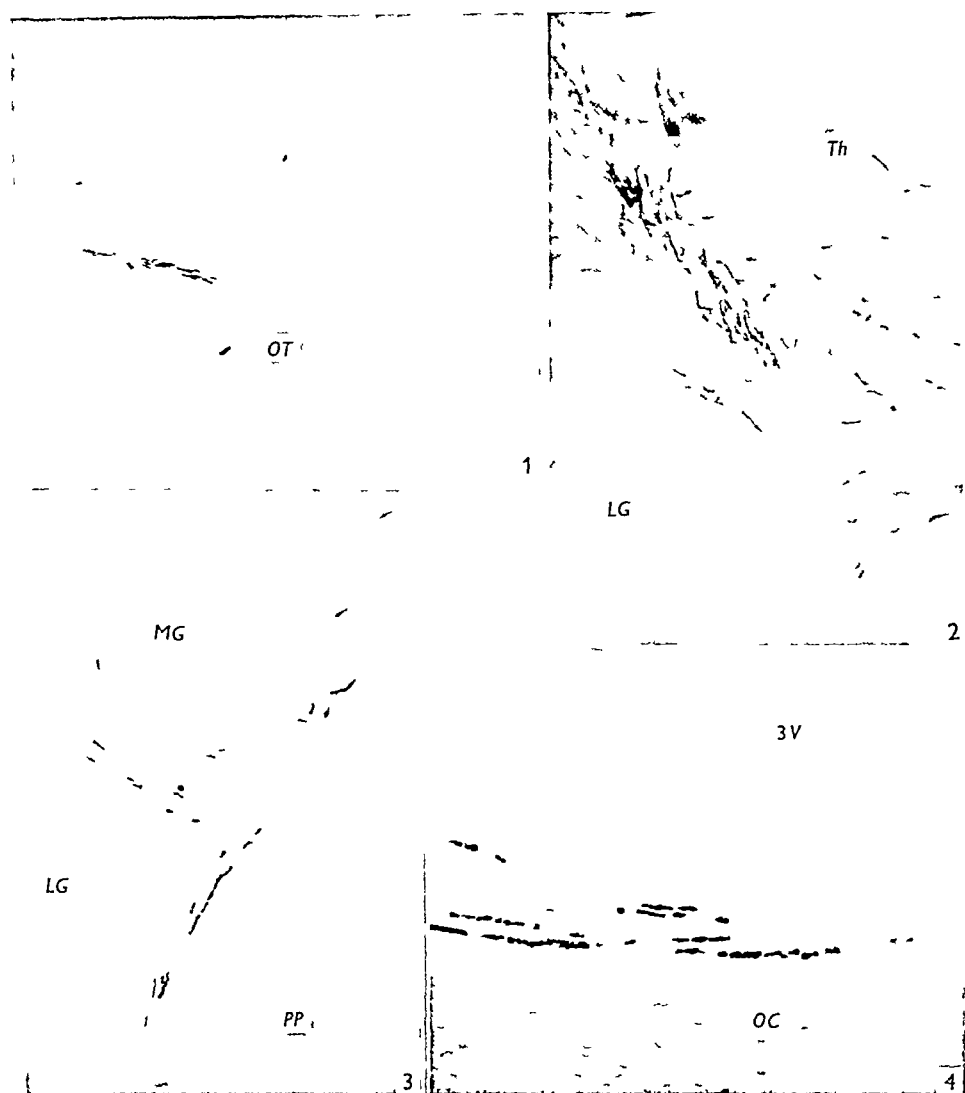
course of the supraoptic fibres of spinal origin in Gudden's commissure, and those leading to the pars ventralis of the lateral geniculate body, with the post-decussational fibres of Gudden's commissure. With respect to the human brain, Gardner & Cuneo (1945), on the basis of Marchi preparations from a patient who died 21 days after an anterolateral chordotomy at the upper thoracic portion of the cord, briefly described degenerating fibres ascending to the medial geniculate body. They did not describe the further course of those fibres. In the light of the present study, it is quite possible that these were supraoptic decussational fibres of spinal origin.

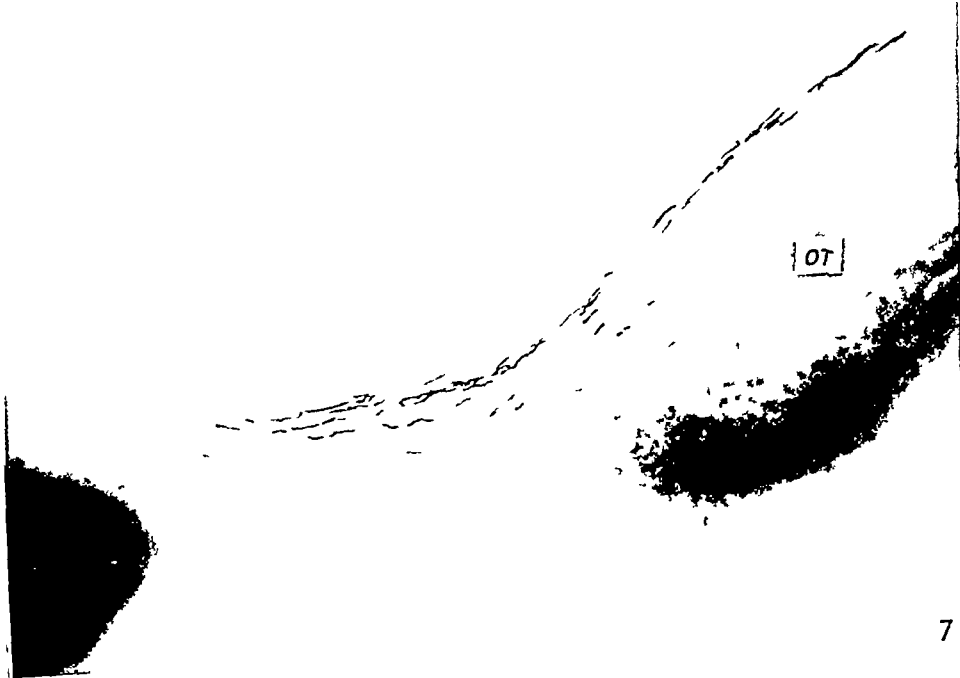
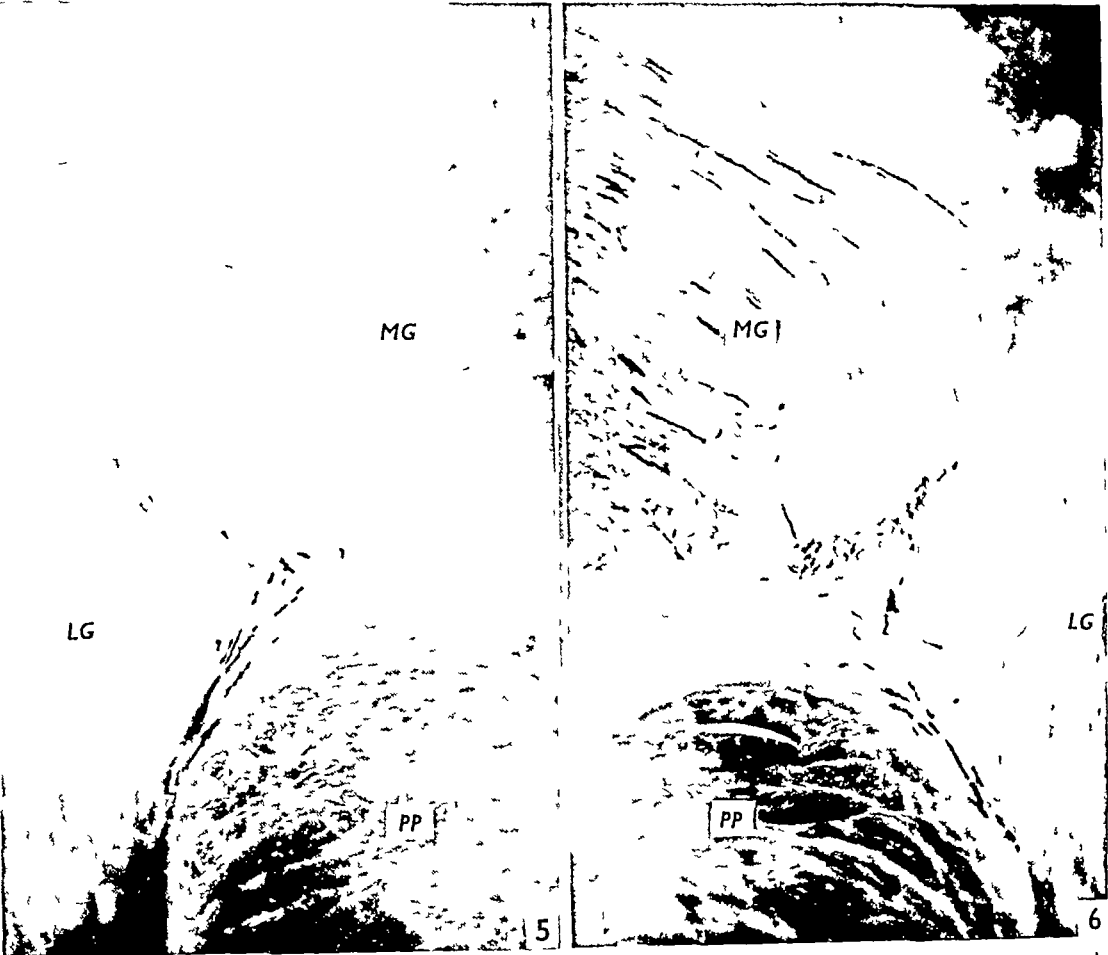
SUMMARY

A previously undescribed component, of spinal origin, in Gudden's commissure was demonstrated by the Marchi method in five spider monkeys. Degenerated fibres were found in Gudden's commissure after transection, hemisection or anterolateral chordotomy at various levels of the spinal cord. Following spinal lesions some of the ascending degenerated fibres in Gowers' fasciculus can be traced upwards to the anterior end of the midbrain where some of them pass through the dorsal aspect of the basis pedunculi, the medial geniculate body, and the space between these two structures to join the medial border of the optic tract. After crossing in the ventral supraoptic decussation of Gudden, the degenerated fibres turn back along the medial border of the optic tract of the opposite side and finally disappear into the region of the nucleus pregeniculatus and the region beneath the medial geniculate body. None of these fibres was observed terminating in the hypothalamus or the globus pallidus, nor did they appear to traverse or to end in the medial geniculate body of the side opposite to the lesion.

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EXPLANATION OF PLATES

All photographs are unretouched

PLATE 1

- Fig 1 Degeneration in the dorsomedial fascicle of the optic tract, spinal transection at Ca_1 (SMS 13) 20× OT=optic tract
- Fig 2 Degenerated fibres in the neighbourhood of nucleus pregeniculatus (SMS 17, after a spinal transection at Ca_1) 20× LG=lateral geniculate body, Th=thalamus
- Fig 3 Degenerated fibres passing over the dorsolateral aspect of the pes pedunculi (SMS 14 spinal hemisection at Ca_1) 20× LG=lateral geniculate body, MG=medial geniculate body, PP=pes pedunculi
- Fig 4 Degenerated fibres in Gudden's commissure (SMS 14, spinal hemisection at Ca_1) 42× OC=optic chiasma, 3V=3rd ventricle

PLATE 2

- Fig 5 Showing the absence of degenerated fibres traversing the medial geniculate body on the left side and the presence of degenerated fibres in the dorsolateral aspect of the pes pedunculi SMS 18 20× LG=lateral geniculate body, MG=medial geniculate body, PP=pes pedunculi
- Fig 6 Taken from the same section as Fig 5, showing the pre decussational stretch of the degenerated fibres passing through the medial geniculate body to the medial border of the optic tract on the operated right side SMS 18 20×
- Fig 7 Section taken at the level of the posterior end of the optic chiasma showing the degenerated fibres in Gudden's commissure after unilateral anterolateral chordotomy at C_2 SMS 18 28×

ALKALINE PHOSPHATASE IN THE UTERINE EPITHELIUM OF THE RAT

By J J PRITCHARD

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INTRODUCTION

Recently it has been shown by a number of workers that the phosphatase activity of certain parts of the reproductive tract is under the control of the gonadal hormones

Thus, in the dog (Huggins, Masina, Eichelberger & Wharton, 1939) and rhesus monkey (Gutman & Gutman, 1939) androgens promote the secretion of acid phosphatase by the prostate. In the castrated mouse the administration of oestrogens leads to a marked increase in the alkaline phosphatase content of the surface and glandular epithelium and circular muscle of the uterus (Atkinson & Elftman, 1946, 1947) and of the vaginal epithelium (Jeener, 1947). In the castrated monkey oestrogens increase and progesterone decreases the alkaline phosphatase activity of the endometrium (Atkinson & Engle, 1947). The same workers showed that this enzyme was present in high concentration in the human endometrium during the proliferative (follicular) phase of the menstrual cycle, but was absent in the secretory (luteal) phase. Pritchard (1948) found that alkaline phosphatase disappeared from the uterine epithelium of the pregnant rat during the active life of the decidua, but reappeared in the latter third of pregnancy.

These observations, though they substantiate the thesis that the phosphatases of the reproductive tract are controlled by the gonadal hormones, give little information as to the precise cytochemical changes involved, or their significance.

It was therefore decided to investigate in detail the distribution and activity of alkaline phosphatase in the uterine epithelium of the rat (a) at different stages of normal reproductive life, (b) following ovariectomy, and (c) following the administration of stilboestrol and progesterone.

It was hoped that by these means the precise effects of the ovarian hormones on the alkaline phosphatase activity of the uterine epithelium might be determined, and a basis provided for further research on the significance of this enzyme in reproduction.

MATERIAL AND METHODS

Ninety-two piebald and albino rats of the Lister strain were used in the investigation. Their uteri were examined for alkaline phosphatase activity at the following stages of normal reproductive life, and after the following experimental procedures:

- (a) Before puberty (seven animals)
- (b) During normal oestrous cycles (twenty-four animals)
- (c) During normal pregnancy (twenty-eight animals)
- (d) During lactation (three animals)

- (e) During pseudopregnancy (one animal)
- (f) During anoestrus (two animals)
- (g) After bilateral ovariectomy (six animals)
- (h) After the administration of stilboestrol (ten animals)
- (i) After the administration of progesterone (eleven animals)

Further details will be found in the section of the paper dealing with results

The timing of the phases of the oestrous cycle was determined from daily, and sometimes twice-daily, vaginal smears. The day after the discovery of spermatozoa in the smear was taken as the first day of pregnancy.

Ovariectomy was performed with aseptic precautions under ether anaesthesia through bilateral dorsal incisions. Both ovaries, uterine tubes, and small portions of the upper ends of the uterine horns, together with surrounding fat, were extirpated.

Stilboestrol was administered in oily solution by subcutaneous injection in single doses which varied from 50 to 400 γ in different animals. Progesterone was administered in similar fashion in single or repeated doses of 5 mg.

Histological technique

In the lactating animals one uterine horn was removed under ether anaesthesia, in the other cases the uteri were removed post-mortem.

The uteri were fixed for 24 hr. in three changes of absolute alcohol, embedded in paraffin, and cut at a standard thickness of 8 μ .

Sections were flattened and dried on the slide with a minimum of heating.

After removal of wax the slides were coated with a thin film of celloidin, passed to water, and then incubated for 2 hr. at 37° C., with frequent agitation, in the sodium glycerophosphate substrate.

The solution used differed slightly from that originally employed by Gomori (1939) and was made up as follows: 2% sodium glycerophosphate (5 parts), 2% calcium nitrate (5 parts), 1% magnesium chloride (2 parts), 0.1% sodium hydroxide (3 parts), distilled water (30 parts). This mixture had a pH of 9.5.

Following incubation the calcium phosphate precipitated at sites of alkaline phosphatase activity was converted into cobalt sulphide in the usual way. A dense black precipitate of cobalt sulphide was interpreted as indicating *intense* alkaline phosphatase activity at the site of deposition, a dark brown precipitate as *moderate* activity, and a light brown one as *slight* activity.

In the description of individual specimens, however, the words 'stained for phosphatase' will be used as meaning 'showed a precipitate of cobalt sulphide' or 'exhibited alkaline phosphatase activity'.

Terminology

In describing the localization of phosphatase activity in glandular and surface epithelial cells, the cytoplasm between the nucleus and the basement membrane will be termed the *basal* cytoplasm, and that between the nucleus and the free surface of the cell, the *distal* cytoplasm. The narrow zone of specialized eosinophilic cytoplasm immediately adjacent to the lumen will be termed the *distal band*.

RESULTS

This paper is primarily concerned with a description of the changes in alkaline phosphatase activity in the epithelial lining of the uterus, and with a correlation of these changes with the physiological activity of the uterus

At one time or another most of the other tissues of the uterus also showed phosphatase activity in greater or lesser degree but, with a few exceptions, physiological correlations were difficult to establish. Only a brief general statement about phosphatase activity in these other tissues will therefore be given before passing to the detailed observations made on the surface epithelium

Muscularis

Ten days after birth (rat 62*a*, Pl 1, fig 1) both circular and longitudinal coats stained for phosphatase. Staining was predominantly nuclear, and the nuclei of the circular coat were more heavily stained than those of the longitudinal coat. At the mesometrial and anti-mesometrial extremities of the uterus the nuclei of the circular coat were particularly heavily stained.

At 21 days (rat 53) staining in these regions was greatly reduced in intensity.

At 28 days (rat 62*b*, Pl 1, fig 2) the muscular coats were completely unstained, except at the mesometrial pole where a small patch of the circular coat showed moderately stained nuclei. All older animals were similar to this one in respect of phosphatase activity in the muscularis.

Stroma

On the basis of phosphatase activity two regions could be differentiated (Pl 1, figs 1, 2). The nuclei of the stromal cells in a zone beneath the surface epithelium and around the glands always showed some activity, which varied in intensity from slight to moderate at different phases of the life history of the animal. The nuclei of the outer stromal cells lying adjacent to the circular muscle coat were always inactive. The stromal cytoplasm was inactive.

There was some evidence that the activity of the nuclei of the inner part of the stroma varied inversely with the intensity of oestrogenic stimulation, but the changes were too slight for this to be claimed with any certainty.

Blood vessels Capillary endothelium invariably showed intense nuclear and cytoplasmic phosphatase activity (Pl 1, fig 4, Pl 2, fig 6). The nuclei in the adventitial coat of the mesometrial arteries were also intensely active in all the specimens examined.

Leucocytes During the normal oestrous cycle polymorphonuclear leucocytes were always present between the stromal cells, and in late oestrus and metoestrus they were present between the epithelial cells also. Their nuclei and cytoplasm always showed intense phosphatase activity.

Glandular epithelium The nuclei always showed moderate to heavy activity (Pl 1, fig 3) in the nuclear membrane, nucleoli and chromatin granules. The basal cytoplasm was invariably inactive, but the distal cytoplasm usually showed diffuse activity, varying in intensity both from cell to cell in the same tubule, and from animal to animal.

In many cells the distal cytoplasmic band showed intense phosphatase activity. The secretion within the glandular lumen was also intensely active.

There was some evidence that phosphatase activity, in nuclei and cytoplasm alike, was increased during oestrogenic stimulation, but as in the case of the nuclei of the stromal cells, the results were not definite enough to establish this with certainty.

SURFACE EPITHELIUM

The uterine cavity of the rat is lined by a single layer of non-ciliated columnar epithelial cells supported, at most times, by a well-marked basement membrane.

The height of the epithelium undergoes marked changes during the oestrous cycle and pregnancy (Allen, 1931), depending chiefly on the level of oestrogenic stimulation (Atkinson & Hooker, 1945). During pro oestrus the height of the epithelium is inversely related to the degree of uterine distention.

In late oestrus the epithelium undergoes degenerative changes associated with the appearance of large vacuoles in the cytoplasm, and polymorphonuclear leucocytes between the cells (Long & Evans, 1922). The basement membrane is lost at this stage.

During metoestrus the epithelium is repaired and mitotic figures make their appearance. These are seen again in the later stages of dioestrus.

Secretory activity is high during pro-oestrus when the secretion distends the uterus to about three times its ordinary size.

These morphological changes in the epithelium, while insufficient for certain diagnosis of a particular phase of the oestrous cycle, provide a useful check on the deductions made from the vaginal smear. For this reason an estimate of the height of the epithelium (obtained by averaging six measurements made with a micrometer eyepiece on portions of the epithelium selected at random) will precede the description of phosphatase activity in each specimen, and the presence of vacuolation, leucocytic infiltration or secretory activity will be noted wherever these phenomena were observed.

Generally, the nuclei and distal cytoplasm of the surface epithelium stained for phosphatase. The basal cytoplasm was always unstained. The distal cytoplasm, apart from the distal band, stained diffusely and rather unevenly in most specimens. The distal band, when stained, appeared homogeneous, but close inspection showed that in reality it was finely granular.

Before puberty

Ten days after birth (rat 62*a*, Pl 1, fig 1, Pl 2, fig 7) the uterine epithelium averaged 11μ in height. Neither nuclei nor cytoplasm stained for phosphatase after the standard incubation time (2 hr).

At 3 weeks (rat 53) and 4 weeks (rat 62*b*, Pl 1, fig 2, Pl 2, fig 8) the height of the epithelium was unchanged, but now the nuclei were faintly stained for phosphatase, although the cytoplasm remained unstained.

At 6 weeks, in one specimen (rat 54, Pl 2, fig 9), the epithelium was 16μ in height, in another (rat 71) it was still only 11μ . In both specimens the nuclei stained faintly. Near the free border of the cells the cytoplasm showed a thin but distinct

It was concluded from this series of observations (1) that during pro-oestrus and early oestrus the distal band shows great phosphatase activity which gradually diminishes during the later stages of the cycle, and almost disappears during anoestrus, (2) that during the stage of uterine distention (late pro-oestrus and early oestrus) great quantities of phosphatase are secreted into the uterine lumen, and (3) that phosphatase activity in the nucleus and distal cytoplasm (excluding the distal band) varies inversely with that of the distal band, being lowest during pro-oestrus and highest during dioestrus

PREGNANCY

The uterine epithelium during pregnancy was studied in two series of rats. In the first series one rat was killed on each day of pregnancy up to full term. In the second series fewer rats were employed and these were killed at intervals of 3 or 4 days.

First 4 days From the time of copulation up to and including the 4th day the changes in the epithelium, both as regards morphological characters and phosphatase activity, were similar to those of late oestrus, metoestrus and dioestrus already described.

5th–12th days On the 5th day (rat 58) the epithelial height had fallen to 10μ , except at the mesometrial end of the lumen where taller cells were found. In this latter situation the nucleus and distal cytoplasm stained faintly for phosphatase, and a thin, moderately heavily stained distal band was present. In the rest of the epithelium the cells did not stain at all. Rat 5 was similar.

On the 6th day (rat 43) the epithelium had a similar height. The nuclei everywhere stained faintly, but the cytoplasm, including the distal band, was unstained.

On the 8th day (rat 87) the epithelium was a little taller (15μ), but neither nuclei nor cytoplasm was stained (Pl 3, fig 17).

From the 9th until the 12th day the chief changes noted were an increasing irregularity of the outline of the lumen and a 'hillocky' appearance of the epithelium. The cells remained unstained.

13th day to full term (21st day) On the 13th day (rat 97) the epithelium was 15μ in height. Both nuclei and cytoplasm were faintly stained for phosphatase. A thin intensely stained distal band was present in about one-half of the cells. In rat 13, at the same day of pregnancy, the epithelium was similar except that the distal band was unstained.

On the 14th day (rat 14) the distal band stained intensely in all cells.

On the 15th day (rat 15, Pl 3, fig 18) the epithelium was taller (20μ). The nuclei were, for the most part, at the basal end of the cells as in pro-oestrus. Both nuclei and distal cytoplasm were moderately heavily stained. The distal band, however, was very wide and stained intensely. Moreover, great quantities of the enzyme were seen in process of secretion into the uterine lumen, again suggestive of pro-oestrus or early oestrus.

There were similar appearances in the epithelium throughout the remainder of pregnancy.

Thus during pregnancy the uterine epithelium shows (1) normal oestrous and dioestrous appearances during the first 4 days, (2) virtual disappearance of the enzyme between the 5th and 12th days, and (3) reappearance of the enzyme on the

13th day, with great activity in the distal band, and great secretory activity, lasting until full term

LACTATION

Three animals only were investigated during lactation. On the 4th day (rat 113) the epithelium was similar to that seen in late dioestrus and on the 4th day of pregnancy.

On the 8th day (rat 115, Pl 4, fig 20) the epithelium was tall (20μ) and the nuclei were regularly arranged in the *distal* part of the cell. The nuclei stained moderately heavily as did the thin strip of distal cytoplasm between the nucleus and the distal band. The latter was fairly wide, and heavily stained, though not so intensely as in pro-oestrus.

On the 15th day (rat 116) the epithelium was not quite so tall (16μ), but the position of the nuclei, and the phosphatase staining, were comparable with those of the previous specimen.

PSEUDOPREGNANCY

This condition was not deliberately investigated, but in one animal (rat 81), which did not show cyclical variations in the vaginal smear, the nuclei of the stromal cells were large, spherical, and vesicular, as in early pregnancy and pseudopregnancy. The animal was not pregnant, and it was assumed that it was pseudopregnant, probably as the result of accidental stimulation of the cervix during the routine vaginal examination.

The uterine epithelium was moderately tall (15μ). The nuclei stained very faintly, and the cytoplasm, including the distal band, was quite unstained (Pl 4, fig 19). The epithelium was therefore similar to that of animals between the 6th and 12th days of pregnancy.

OVARECTOMY

The uterine epithelium was examined in bilaterally ovariectomized animals at intervals from the 8th day until 4 months after operation.

On the 8th day (rat 59, Pl 4, fig 21) the epithelium was 10μ in height. The nuclei were moderately heavily stained. The distal cytoplasm stained faintly apart from the distal band, which was thin, but heavily stained, in some cells. Other cells showed either faint staining or complete absence of staining in the distal band. Similar appearances were seen on the 9th day (rat 100).

At the 15th day (rat 60) the epithelium was 9μ in height. While the nuclei and general cytoplasm stained as before, the distal band was very thin and stained with only moderate intensity. In some cells it was absent altogether.

Rat 68, at the 14th day, was similar.

Little change was seen either in the morphological characters or in the phosphatase staining of the epithelium, at longer intervals after operation. After 4 months (rat 63, Pl 4, fig 22) the nuclei stained with faint to moderate intensity, and the distal band, though extremely thin, still stained heavily.

Thus, following ovariectomy, there is a marked reduction in phosphatase activity in the uterine epithelium, although some activity persists for at least 4 months. Fifteen days after ovariectomy the epithelium is very similar to that seen in anoestrus and in animals about 6 weeks of age.

STILBOESTROL

Immature animals In rat 71, 6 weeks after birth the uterine epithelium was 11μ high, the nuclei and distal cytoplasm stained faintly and a thin moderately heavily stained distal band was present

Rat 71a, a litter mate, was given 50γ stilboestrol in oil by subcutaneous injection After 24 hr its uterine epithelium was 26μ in height The nuclei and distal cytoplasm stained moderately heavily The distal band was very much wider than in the control, and stained intensely, and some heavily stained secretion was present within the lumen

Rat 72, another litter mate, was given a similar dose of stilboestrol Forty-eight hours later the uterus showed moderate pro-oestrous distention and its epithelium was very tall (30μ) The nuclei and distal cytoplasm were less heavily stained than in the previous specimen, but the distal band was even wider, and great quantities of secretion were seen passing into the uterine lumen (Pl 4, fig 23)

Rat 72a, yet another litter mate, was given a similar dose of stilboestrol Four days later the uterus was undistended and its epithelium was 24μ in height, and showed commencing vacuolar degeneration The nuclei and distal cytoplasm stained moderately heavily, and the distal band was wide and intensely stained, but there was little secretory activity

Ovariectomized animals In rat 69, 6 weeks after bilateral ovariectomy, the epithelium was 7μ in height The nuclei stained faintly, the distal cytoplasm was unstained, and the distal band, when present, was very thin and only moderately heavily stained Rat 70, ovariectomized for a similar length of time, was given 50γ stilboestrol Forty-eight hours later the uterus was moderately distended and the epithelium was 25μ in height (Pl 4, fig 24) The nuclei stained with faint to moderate intensity, and the distal cytoplasm was faintly stained The distal band was wide and very heavily stained and there was great secretory activity

Rat 70a was treated similarly, except that it was examined 4 days after the injection of stilboestrol The uterus was undistended and vacuolar degeneration and leucocytic infiltration of the epithelium were marked The cells were 25μ high The nuclei and distal cytoplasm were moderately heavily stained The distal band was wide, and intensely stained, but secretory activity was minimal

Rat 51, ovariectomized 3 months previously, was given 200γ stilboestrol Four days later the uterus was undistended The epithelium was extremely tall (40μ) and there was no sign of vacuolar degeneration or leucocytic infiltration The nuclei stained very faintly and the cytoplasm was unstained In some cells the distal band was wide and intensely stained, but in others it was unstained Very great quantities of heavily stained secretion, however, were present in the lumen Rat 64, also ovariectomized 2 months previously, was given 400γ stilboestrol Four days later the uterine epithelium was in a similar condition to that just described

Rat 67, ovariectomized 24 days previously, was given 200γ stilboestrol Three days later the uterus was extremely distended and the epithelium was only 7μ in height The nuclei stained faintly, but the cytoplasm, including the distal band, was completely unstained Great quantities of secretion, however, were present within the lumen

Thus 50 γ stilboestrol injected into either immature or ovariectomized rats, reproduces pro oestrous and oestrous conditions in the uterine epithelium, both as regards morphological and phosphatase characters. With larger doses the enzyme disappears completely from many of the cells, presumably because it has all been secreted into the lumen.

PROGESTERONE

Rat 111 was found to be in pro-oestrus at the time of the routine vaginal smear. 5 mg. of progesterone in oil were injected subcutaneously and the animal was killed 2 days later. The uterine epithelium was 16 μ in height. The nuclei and distal cytoplasm stained moderately heavily, and the distal band was moderately wide and intensely stained. The cells, in fact, stained as in early dioestrus.

Rat 101, also in pro-oestrus, was given 5 mg. progesterone and a similar dose 2 days later when killed. Five days after the first injection the epithelium was 14 μ in height. The nuclei stained extremely faintly, and the cytoplasm, including the distal band, was completely unstained (Pl. 4, fig. 25).

Rat 112 was also in pro-oestrus. It was treated in a similar manner to the last named, except that it was not killed until 8 days after the first injection. The epithelium was 15 μ high, and no phosphatase was detectable in either nucleus or cytoplasm.

Rats 120, 121 and 122 were treated similarly at pro-oestrus. They were killed 11 days after the first injection. Rat 120 showed similar appearances to those just described. Rats 121 and 122, however, were in pro-oestrus and showed the characteristic wide heavily stained distal band and minimally stained nucleus and general cytoplasm.

Evidently progesterone causes the complete disappearance of phosphatase from the nucleus and cytoplasm, the effect beginning to wear off about 9 days after the last injection.

Rats 50 and 66 were injected with 5 mg. progesterone 3 months after ovariectomy, and killed 5 days later. The epithelium showed no significant differences from that of untreated ovariectomized animals, so it would appear that progesterone leads to a disappearance of epithelial phosphatase only when it acts on cells recently under the influence of oestrogens.

For convenience of cross-reference the more important observations on the morphological characters and phosphatase activity of the uterine epithelium at the various stages detailed above have been summarized and tabulated (Table 1).

Table 1

Stage or experiment	Epithelial height (μ)	Nuclear phosphatase activity	Distal cytoplasmic phosphatase activity	Distal band phosphatase activity	Secretory activity	Remarks
Before puberty						
10 days	11	0	0	0	0	—
21 days	11	+	0	0	0	—
28 days	11	+	0	0	0	—
6 weeks	11-16	+	\pm	+	0	—
8 weeks	23	+	\pm	—	0	—
13 weeks	20	++	+	++	0	Most animals of this age undergoing oestrous cycles

Table 1 (*continued*)

Stage or experiment	Epithelial height (μ)	Nuclear phosphatase activity	Distal cytoplasmic phosphatase activity	Distal band phosphatase activity	Secretory activity	Remarks
Anoestrus	15	++	+	\pm	0	—
Pro oestrus						
Early	30	\pm	0	++++	+	Regularly arranged basal nuclei
Middle	25	+	\pm	++++	+++	—
Late	8	++	+	++++	++++	Grossly distended uterus
Oestrus						
Early	8	++	+	++++	++++	Grossly distended uterus
Middle	15	++	+	++++	++++	—
Late	27	++	++	+++	++	Vacuolar degeneration
Very late	30	++ to +++	++	++	+	Vacuolar degeneration and polymorphic in filtration
Metooestrus	19	++	++	++	0	Golgi element heavily stained
Dioestrus						
Early	19	++	++	++	0	—
Late	15	++	+	+	0	—
Pregnancy						
0-4 days	—	—	—	—	—	Similar to late oestrus, metooestrus and dioestrus
5-12 days	10-15	\pm	0	0	0	—
13-21 days	15-20	+ to ++	+ to ++	++++	++++	—
Lactation						
4th day	—	—	—	—	—	Similar to dioestrus
8th day	20	++	++	++	0	Nuclei distal
15th day	16	++	++	++	0	Nuclei distal
Pseudopregnancy	15	\pm	0	0	0	—
Ovariectomy						
8 days	10	++	\pm	0 to +	0	—
15 days	9	++	\pm	0 to +	0	—
4 months	7	+ to ++	\pm	+	0	—
Stilboestrol to immature rats						
50 γ 24 hr	26	++	++	+++	++	—
50 γ 48 hr	30	+	+	++++	+++	—
50 γ 96 hr	24	++	++	++++	+	Vacuolar degeneration
Stilboestrol to ovariectomized rats						
50 γ 48 hr	25	+ to ++	+	++++	+++	—
50 γ 96 hr	25	++	++	++++	\pm	Vacuolar degeneration and leucocytic in filtration
200-400 γ 96 hr	40	\pm	0	0 to ++	++++	No degeneration
Progesterone at pro oestrus						
5 mg 48 hr	16	++	++	++	0	—
10 mg 5 days	14	\pm	0	0	0	—
10 mg 8 days	15	0	0	0	0	—
10 mg 11 days (a)	15	0	0	0	0	—
10 mg 11 days (b)	30	\pm	\pm	++++	++	Effect worn off
Progesterone to ovariectomized animals	—	—	—	—	—	Animal pro oestrous again
						Same as untreated controls

0, no phosphatase activity, \pm , minimal activity, +, slight activity, ++, moderate activity, + + +, heavy activity, + + + +, very heavy activity

DISCUSSION

This investigation has shown that the distribution and intensity of alkaline phosphatase activity within the surface epithelium of the uterus of the rat exhibits gross variations during normal reproductive life, following castration, and after the administration of stilboestrol and progesterone. Variations in the activity of the enzyme within the other tissues of the uterus are of a minor character, and of doubtful significance.

In the surface epithelium the alkaline phosphatase activity of the distal band of the cytoplasm shows the greatest variation. The changes in the nucleus and distal cytoplasm, apart from the distal band, are definite but much less pronounced. The basal cytoplasm never shows phosphatase activity. Maximal activity in the distal band is associated with secretion of the enzyme into the uterine lumen.

The changes in cytoplasmic activity are largely independent of those occurring in the nucleus. This is not surprising for, as was discussed in a previous paper (Pritchard, 1948), the cytoplasmic phosphatases appear to be chiefly concerned with functional changes in mature cells such as are involved, for example, in secretory activity, while the nuclear phosphatases are more concerned in the metabolic exchanges associated with cell division and differentiation.

The results summarized in Table 1 show that the effects of hormonal administration and ovariectomy on the distribution and activity of the enzyme in the uterine epithelium are closely paralleled by the changes occurring at certain stages of normal reproductive life.

Thus stilboestrol produces changes in the phosphatase activity of the uterine epithelium virtually identical with those found at pro-oestrus and in late pregnancy, when there is every reason for supposing that the endogenous production of oestrogens reaches a high level (Selye & McKeown, 1935). Progesterone, on the other hand, reproduces the conditions found in early pregnancy and pseudopregnancy when the endogenous production of progesterone is known to be at a high level (Atkinson & Hooker, 1945). There can be little doubt, therefore, that the naturally occurring variations in phosphatase activity during the oestrous cycle and in pregnancy are determined by the levels of circulating oestrogens and progesterone.

Furthermore, the phosphatase activity of the uterine epithelium in the sexually immature animal is consistent with a rising but sub-threshold oestrogen level, and that of the anoestrous and castrated animal with a very low oestrogen level, which is in accordance with what might have been expected in view of the conclusion reached above.

The phosphatase activity of the epithelium during lactation, however, appears at first sight to be at variance with what might have been expected. Judging from the level of phosphatase activity alone, a moderate level of oestrogenic activity might be inferred.

The case with which deciduomata may be produced during lactation in the rat (Corner & Warren, 1919, Long & Evans, 1922, Selye & McKeown, 1935, Lyon & Allen, 1938) suggests that the level of circulating oestrogen is very low. In the mouse also there is evidence that the oestrogen level is very low during lactation (Atkinson & Leatham, 1946).

It is probable, however, that the production of oestrogens during lactation varies from animal to animal. For instance, Crew & Mirskaia (1930) state that while oestrous cycles are normally in abeyance during lactation, they may be resumed if the number of young suckled is small. Again, Krehbiel (1941) found that, depending on the size of the litter, a pregnancy of normal duration may accompany lactation in some cases, in others implantation may be delayed and pregnancy prolonged, in others again pregnancy may fail with absorption of embryos. In this connexion it may be significant that the rats employed in this investigation all had small litters, which would be compatible with a moderate level of circulating oestrogen as suggested by the level of phosphatase activity.

The functional significance of these changes in uterine phosphatase activity has not yet been determined. The fundamental role of the phosphatases is no doubt that of assisting in the hydrolysis of organic phosphate esters (Moog, 1946), but as this type of chemical change occurs during the intermediary metabolism of carbohydrates, fats and proteins alike, and probably also in the transport of a wide variety of organic substances, as well as in the early stages of calcification, it is evident that the exact significance of variations in phosphatase activity cannot be inferred from purely histological studies. One can only hope that the results obtained by histochemical studies such as this will point the way to significant biochemical investigations. There is, for example, possibly a physiological significance in the fact that oestrogens cause not only a mobilization of phosphatase in the uterine epithelium of the rat but also of glycogen (Boettiger, 1946), while lipoids are sharply diminished (Bourg, 1930, Alden, 1946). Again, the secretion of great quantities of the enzyme into the distended lumen at oestrus may be associated in some way with the metabolism or motility of spermatozoa.

Whatever the underlying explanation may turn out to be, the fact that phosphatase activity in the uterus is so closely linked with ovarian function, makes it probable that the role of the enzyme in reproductive processes is an important one.

SUMMARY

1 The alkaline phosphatase activity of the uterine epithelium of the rat has been investigated histochemically in the immature animal, throughout the oestrous cycle, in pregnancy, pseudopregnancy, lactation, after castration and following the administration of stilboestrol and progesterone.

2 Gross variations in the activity of the enzyme were found, particularly in the distal band of cytoplasm bordering the uterine lumen.

3 The results indicate that these variations are determined by the level of circulating oestrogens and progesterone.

4 Oestrogens cause a great concentration of the enzyme in the distal band of the cytoplasm, and its secretion into the uterine lumen.

5 Progesterone causes the disappearance of the enzyme from all parts of the cell.



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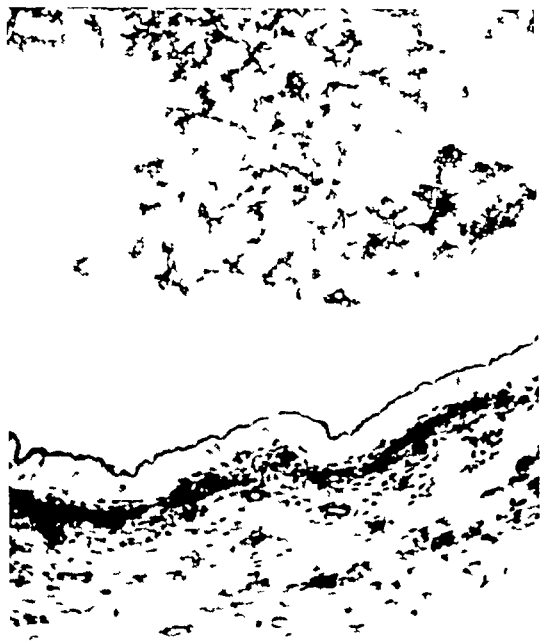
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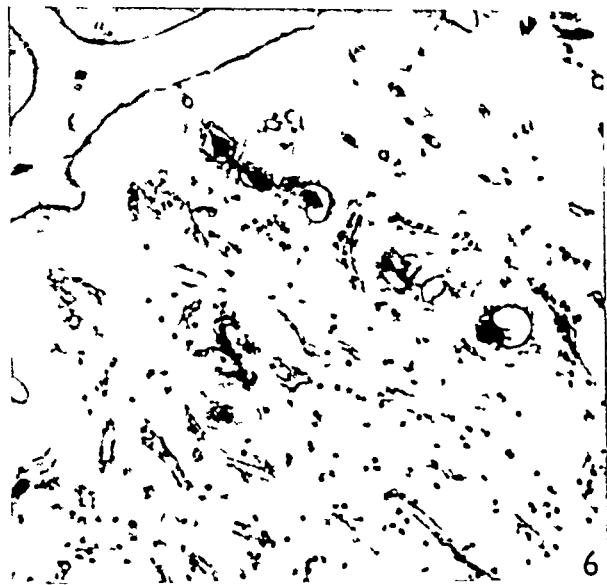
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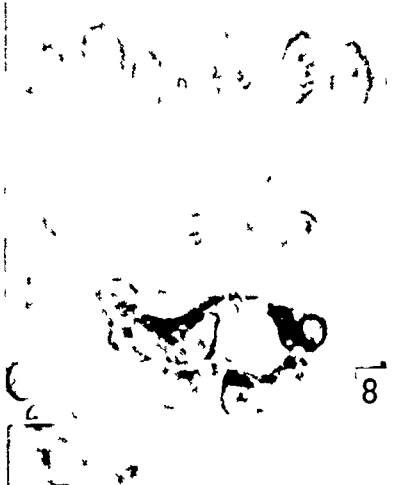
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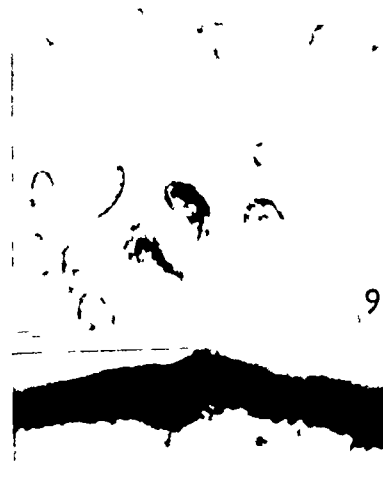
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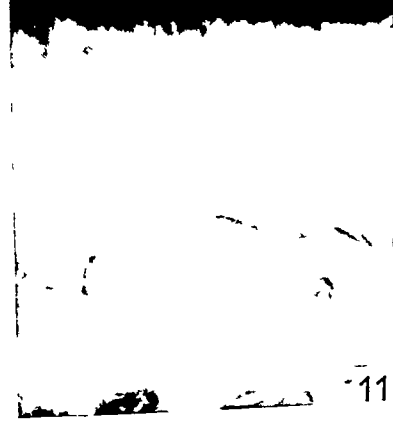
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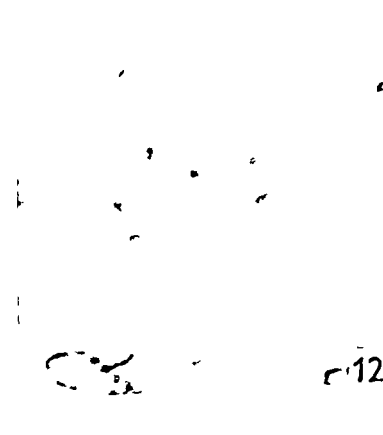
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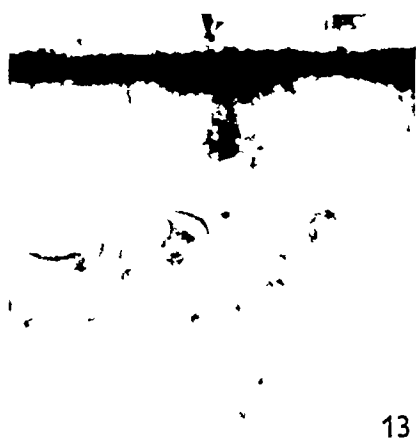
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EXPLANATION OF PLATES

All sections illustrated were stained exclusively for alkaline phosphatase. Figs 1, 2, 5 and 6 are at a magnification of 150. The remainder at a magnification of 1350. In each of these two groups the photographs were obtained under identical conditions of exposure, development, etc. Figs 7-25 show the uterine epithelium with some of the adjacent stroma at various stages of reproductive life and after castration and the administration of stilboestrol and progesterone.

PLATE I

- Fig 1 A transverse section of the uterus of a 10 day old rat
 Fig 2 A transverse section of the uterus of a 28 day old rat
 Fig 3 Part of a uterine gland during oestrus
 Fig 4 Part of the endometrium during oestrus

PLATE 2

- Fig 5 Part of the uterine wall and uterine lumen at pro oestrus
Fig 6 Part of the uterine wall and uterine lumen in early oestrus
Fig 7 Ten days after birth
Fig 8 Twenty eight days after birth
Fig 9 Six weeks after birth
Fig 10 Late pro oestrus, with gross distention
Fig 11 Pro oestrus, with moderate uterine distention
Fig 12 Pro oestrus, before distention is marked

PLATE 3

- Fig 13 Oestrus—stage of vacuolation
Fig 14 Oestrus—stage of leucocytic infiltration
Fig 15 Metoestrus
Fig 16 Late dioestrus
Fig 17 Eighth day of pregnancy
Fig 18 Fifteenth day of pregnancy

PLATE 4

- Fig 19 Pseudopregnancy.
Fig 20 Eighth day of lactation
Fig 21 Eight days after castration
Fig 22 Four months after castration
Fig 23 Six weeks old, 50 γ stilboestrol given 2 days previously
Fig 24 Six weeks after castration, 50 γ stilboestrol given 2 days previously
Fig 25 Five days after pro oestrus, 5 mg progesterone given on the 1st and 3rd days

SOME NEW OBSERVATIONS UPON THE GRANULES OF THE OXYNTIC CELLS OF NORMAL RATS

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INTRODUCTION

Lim & Ma stated in 1926 that the granules of the oxyntic cells of the rat's stomach contained lipid. They showed that these granules were stained by Janus green B, and they therefore took them to be mitochondria, and, believing that all mitochondria contained lipid, they presumed that the granules must contain lipid. They stated, in addition, that the granules could often be impregnated with osmium tetroxide, which they took to be evidence of a lipid content. The argument rests upon three assumptions, viz that Janus green B is a specific stain for mitochondria, that all mitochondria contain lipid, and that osmium tetroxide is a reagent specific for lipids. It is known, however, that osmium tetroxide may be reduced by substances other than lipids (Lison, 1936), and even if Janus green B is specific for mitochondria it has not been shown that all mitochondria contain lipid. It follows that Lim & Ma's observations, though suggestive, do not provide a satisfactory proof of the presence of lipid in the granules of the oxyntic cells. Recently, Foster in this Department has shown (unpublished work) that the granules of the oxyntic cells of the rat's stomach could be stained with Sudan black, suggesting again the presence of a lipid. It was therefore decided to try to investigate further the lipid content of the granules, chiefly by the use of Baker's acid haematein test (1946) for lipines, and by various other methods.

MATERIAL AND METHODS

Twenty-four male Norwegian rats, kept in separate cages, were starved, except for an allowance of water, for 24 hr. They were then killed by coal gas, used in preference to other anaesthetics as being less likely to affect the lipid content of the cells. The stomach was then removed and pieces of the mucous membrane from the fundus were treated as follows.

(a) Fixed in formol-calcium-chloride (Baker, 1944), washed and embedded in gelatin. Sections were then cut at 10μ on the freezing microtome, and coloured with (i) Sudan black (Baker, 1944), (ii) Sudan II, Sudan III, Sudan IV, Nile blue sulphate.

(b) Fixed in formol-calcium-chloride and (iii) Baker's acid haematein test applied (Baker, 1946).

(c) Fixed in the following modification of Bouin's fluid

Saturated aqueous solution of picric acid	50 c c
Commercial formalin	10 c c
Glacial acetic acid	5 c c
Distilled water	35 c c

After fixation the lipoids were removed by hot pyridine (Baker, 1946) and the tissues embedded in gelatin. Sections were cut frozen, at 10μ , and treated as follows (iv) by Baker's acid haematein test, (v) with Sudan black

(d) Lipoids were removed by hot pyridine, after fixation in the modified Bouin's fluid described above. The tissues were then washed and dehydrated, cleared and embedded in wax, and sections cut at 5μ were treated with (vi) Heidenhain's iron haematoxylin

(e) Fixed in Champy's fluid and treated by (vii) the Nassanov-Kolatchew technique, a routine method for the demonstration of the Golgi element by the use of osmium tetroxide

The following inferences may be drawn from certain reactions to the above methods - A positive reaction to Sudan black, or Sudan II, III or IV, indicates the presence of lipid, Sudan black frequently demonstrating lipid when the others fail to do so. A rose or red coloration with Nile blue sulphate, according to Lison (1936), indicates the presence of a non-saturated glyceride. A positive reaction to Baker's acid haematein test shows the presence of lipines and/or several proteins. Hot pyridine, however (as used in the pyridine extraction test), removes lipoids but not the proteins, and thus a subsequent application of the acid haematein test would, if negative, indicate the absence of both the lipoids and the several proteins that react to the test. The combination of a positive reaction to the acid haematein test and a negative reaction following pyridine extraction indicates the presence of lipine alone

RESULTS

(1) *With Sudan black* (Baker, 1944) (Pl 1, fig 1) The granules in the oxyntic cells colour intensely, appearing a bluish black. There are many granules in the cells, some being quite discrete, but the majority are not very sharp in outline. They vary in size, the largest being approximately twice the diameter of the smallest visible, and there are many of intermediate size. There is some clumping of the granules. The nucleus is pale and unstained, but the cytoplasm is grey in patches, indicating the presence of a diffuse lipid substance. The patchy greyiness of the cytoplasm probably accounts for the fact that the majority of the granules do not appear to have a sharp outline. Completely uncoloured areas devoid of granules can frequently be seen marking the position of intracellular canals.

(2) *With Sudan II, III and IV and Nile blue sulphate* No positive reaction is given by the granules in the oxyntic cells, and there is no indication of a rose or red colour with Nile blue.

(3) *With acid haematein* (Baker, 1946) (Pl 1, fig 2) The granules stain black or blue-black. They are numerous and discrete, and more definite than with

Sudan black The cytoplasm is colourless, and this may account for the discrete appearance of the granules. In positively reacting cells no difference is apparent in the intensity of colouring between those deeply situated and those superficially placed in the tubule, or between cells in one tubule and another, but a few oxyntic cells in the depths of the glands show no positive reaction. It seems unlikely that this is due to a failure of penetration of the reagents, for these cells occur at random, surrounded by positively reacting cells. Except in the negatively reacting cells, there are no unstained refractile granules visible. The granules are dispersed evenly throughout the cytoplasm, save where colourless channels representing the intracellular canals can be seen, and in a few areas there is some clumping of the granules. The granules vary in size within the same cell, the largest having a diameter approximately twice that of the smallest visible. They are all spherical. Some granules are more deeply coloured than others, some appear quite black, some blue-black. It is frequently difficult to tell whether the darkness of the granules is due to clumping and consequent overlapping, or to their size. On the whole the depth of staining is uniform, and where some granules appear more deeply coloured than others this can frequently be shown by careful focusing to be due to the fact that one granule is lying above another.

(4) *With acid haematein following pyridine extraction of lipoids* (Baker, 1946) (Pl 1, fig 3) None of the granules in any of the oxyntic cells appears coloured, but they can still be seen by virtue of their refractility. The nucleus stains deeply, as also do the red blood corpuscles (see Baker, 1946).

It may be mentioned here that there are small cells in the connective tissue of the submucosa whose granules are positive to acid haematein and negative after pyridine extraction, although their significance is not known.

(5) *With Sudan black following pyridine extraction of lipoids* None of the granules of the oxyntic cells is coloured. This is what one would expect, the test being applied merely as a check on the acid haematein test.

(6) *With iron haematoxylin following pyridine extraction of lipoids* (Pl 1, fig 4) The granules stain a dark greyish brown and appear in about equal numbers in all cells of similar size, and there appear to be as many granules per cell as in comparable cells to which the acid haematein test has been applied without pyridine extraction.

(7) *With the Nassanov-Kolatchew technique for the Golgi element* All oxyntic cells show a dense nuclear membrane, and, in addition

(a) The majority show slightly osmophil granules spread throughout the cytoplasm, and indistinct osmophil strands in the cytoplasm between the granules.

(b) The minority show a definite osmophil network spreading throughout the cytoplasm. This network, which is slightly more osmophil than the granules described above, is closely applied round refractile unstained granules.

(c) A few cells show a collection of very dark osmophil strands fairly well localized to one part of the cell.

Many mucous and peptic cells show a typical Golgi element—a localized net between the nucleus and the gland lumen, the oxyntic cells do not.

DISCUSSION

The conclusions arrived at by Lim & Ma (1926) have been confirmed—Sudan black shows that the oxyntic granules contain lipid. Furthermore, the acid haematein and pyridine extraction tests show that they contain lipine, presumably a phospholipine, since galactolipine is believed to occur only in the central nervous system. Sudan black indicates also the presence of a diffuse lipid in the cytoplasm.

The application of iron haematoxylin, after the lipoids have been removed by pyridine, shows that a stainable residue is present, probably protein or partly protein in nature. It can therefore be inferred that the granules are of a double nature, partly lipid and partly non-lipid. It is probable that the lipid forms an envelope to a non-lipid core, though the evidence is not yet adequate to decide this point.

There are other cell inclusions having lipid and non-lipid components, namely, (1) mitochondria (Claude, 1941, Bourne, 1942, Hoerr, 1943, Baker, 1946), and (2) the Golgi element (Hirsch, 1939, Baker, 1944, Foster, 1947).

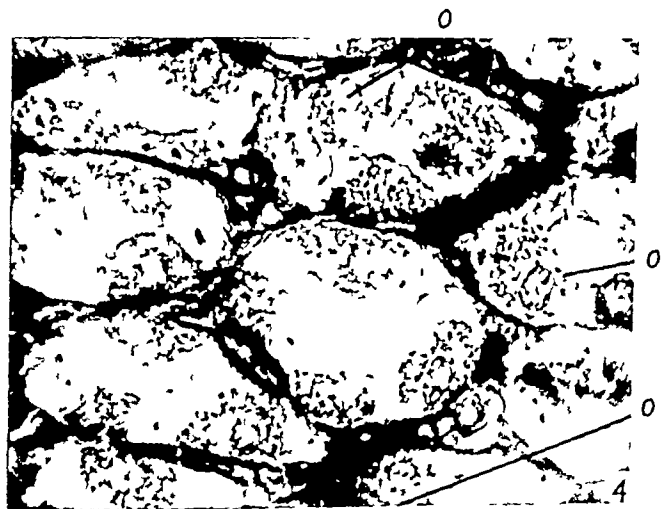
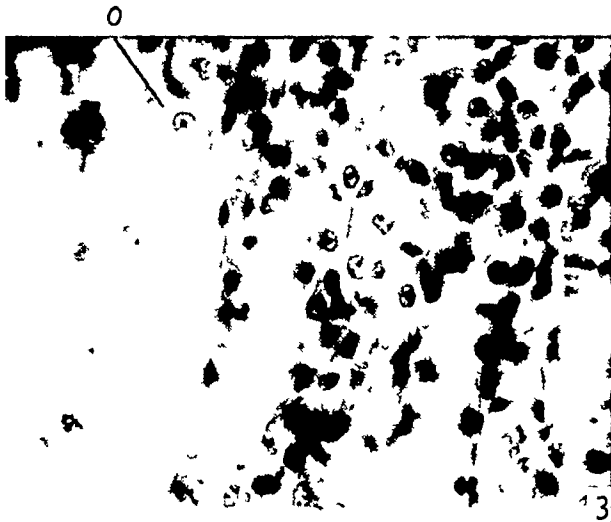
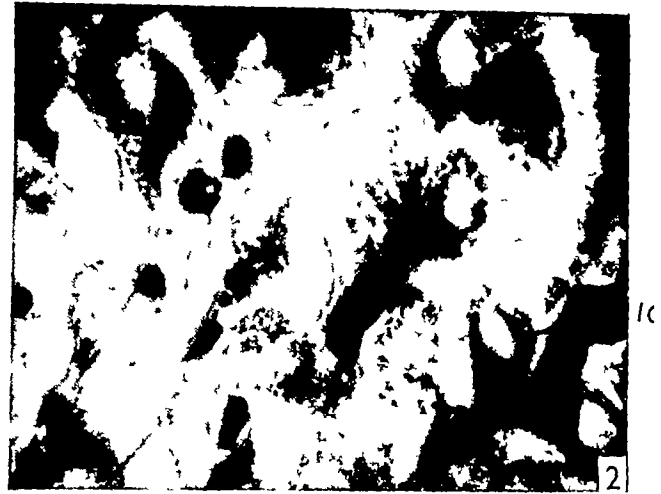
Kolster in 1913 stated that there were two types of Golgi element in the oxyntic cell, one completely filling the cell, the other being more localized. Both Siraska (1929) and Shiraska (1930) denied the existence of Golgi material in this cell. In 1932 Beams & King investigated the Golgi element in the oxyntic cell of the rat, and although they never found as complete a network as reported by Kolster, they described it as being present in the form of robust filaments scattered throughout the cytoplasm. In my preparations using the Nassanov-Kolatchew method, both types seen by Kolster are undoubtedly present, together with a third in which the Golgi element is seen as black granules with osmophil strands in the cytoplasm between them. I would suggest that the dissimilarities in the three types of Golgi element seen are due to different functional states of the cells concerned.

A connexion may be seen here between the Golgi element, as shown in the preparations mentioned above, and those parts of the oxyntic granules proved to be lipid. For, supposing that the lipid in all the granules represented the osmophil part of the Golgi element, then one would expect to find a very large Golgi element, or a continuous net, occupying the greater part of the cell, and such is often the case. Where, as is sometimes observed, the Golgi element occupies only a small proportion of the cell, then, on the hypothesis just suggested, this could mean that a smaller number of the lipid-containing granules reduced the osmium tetroxide, and perhaps it is these that are observed in some cells to be more deeply coloured with acid haematein than the rest.

SUMMARY

1 The granules of the oxyntic cell of the rat's stomach contain lipid, as shown by their intense colouring with Sudan black.

2 They contain lipine, probably phospholipine, demonstrated by a positive reaction to Baker's acid haematein test, and a negative reaction to this test following the removal of lipoids by hot pyridine.



3 They are of a double nature, possessing a core that is neither lipine nor lipid, since they are stainable by iron haematoxylin after the lipid component has been removed

4 There is some evidence that the cytoplasm of the oxyntic cell contains a diffuse non-lipine lipid material It is indicated by Sudan black

I am greatly indebted to Dr C L Foster, who suggested this line of research to me, and I wish to express my thanks to him and to Prof F Goldby, for their continued interest and help in this work

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EXPLANATION OF PLATE

- Fig 1 Fundic area of rat's stomach coloured with Sudan black, $\times 500$
- Fig 2 Granules in oxyntic cells, after application of Baker's acid haematein test $\times 1050$
- Fig 3 Absence of colouring of granules when Baker's acid haematein test is applied following pyridine extraction of lipid, $\times 500$ N B The oxyntic nuclei are now coloured (see Baker, 1946)
- Fig 4 Appearance of oxyntic granules after staining with Heidenham's iron haematoxylin, following pyridine extraction according to Baker's method, $\times 950$
- O = oxyntic cell IC = site of an intracellular canal Figs 1-3 are cut along the tubules of the fundic glands Fig 4 is cut across the tubules

A NEW HISTOCHEMICAL METHOD FOR GLYCOGEN

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The method to be described is based first on Bauer's (1933) observation that glycogen, after treatment with chromic acid, is rendered insoluble in water and exhibits the reactions for aldehydes, and secondly on Mitchell & Wislocki's (1944) observation that under certain conditions glycogen will reduce ammonium-silver compounds. The following method is the outcome of experiments made to determine whether the specificity of Bauer's method could be associated with the sharpness of definition and ease of photographic reproduction of Mitchell & Wislocki's method, and it was found that glycogen in tissue sections was capable of reducing ammonium-silver compounds specifically after treatment with chromic acid. Exceptionally clear and detailed pictures of glycogen distribution in a number of tissues were obtained.

METHOD

Tissues are fixed in ice-cold absolute alcohol for 24-48 hr and then embedded in paraffin in the usual way. Sections are cut and flattened on 70 % alcohol and dried on the slide without the use of adhesives. After removal of wax they are coated with celluloid by immersion in 0.5 % solution of celluloid in alcohol-ether followed by hardening in 70 % alcohol.

After passing to distilled water, the sections are placed in 4 % chromic acid at 55° C for 25-30 min and then washed well in distilled water. They are transferred to Foot's silver diamino hydroxide solution for 2-5 min and then, without washing, immersed in Wilder's formol-uranium reducing solution, with agitation, for 2 min.

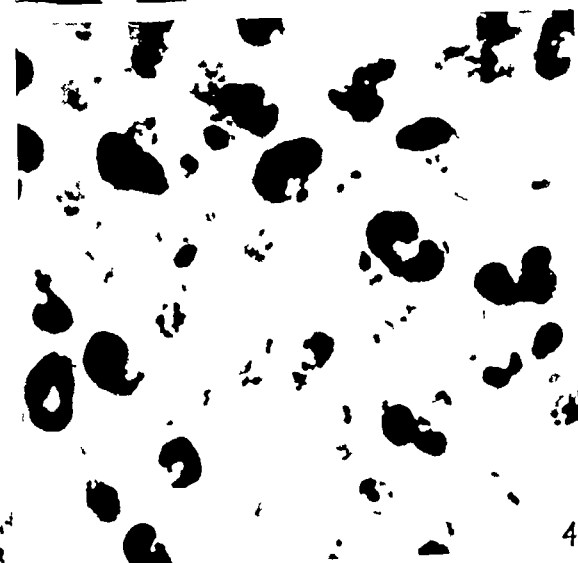
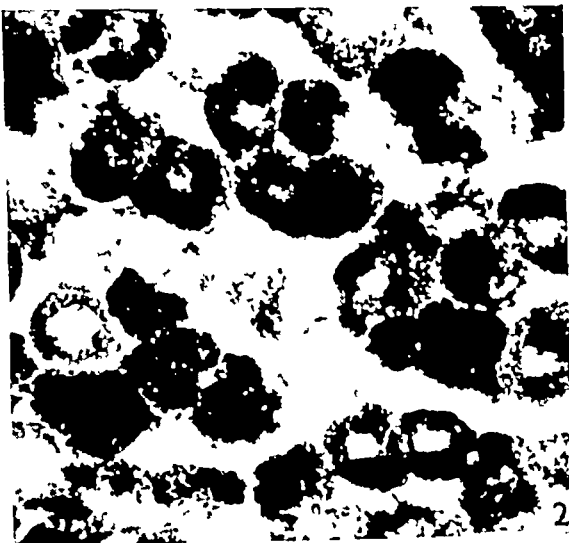
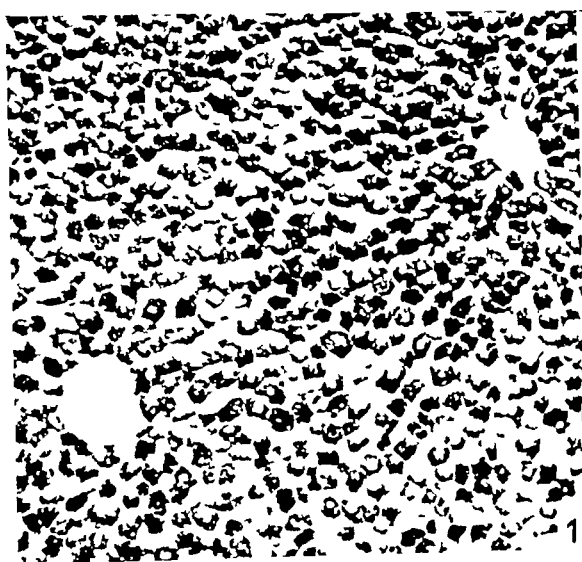
They are again washed in distilled water and toned for 2-5 min in 0.2 % gold chloride solution, rinsed in tap water and placed in 5 % sodium thiosulphate solution for 2 min.

After a further wash in distilled water the sections may be counter-stained (light green or acid fuchsin are suitable) and mounted.

Glycogen is stained purplish black by this method, and the background is clear. No other tissue components take the stain.

That the granules stained by this method consisted of glycogen was confirmed by the saliva test, which is based on the fact that glycogen is the only polysaccharide present in animal tissues which is digested by salivary ptyalin. After the sections had been incubated for 30 min in a 25 % solution of saliva in tap water at 37° C no reduction of silver occurred after subsequent treatment with chromic acid and silver diamino hydroxide.

Pl 1, figs 1-4 illustrate examples of the results obtained.



DISCUSSION

The method described differs from that of Bauer (1933) in the substitution of silver diamino hydroxide for sulphuretted basic fuchsin (Feulgen's reagent) as the means of visualizing glycogen after treatment with chromic acid. This has the advantage that the precipitate of reduced silver is more sharply defined, particularly in relation to very small particles of glycogen, than the basic fuchsin stain, it can also be photographed much more easily and with better contrast.

Mitchell & Wislocki's (1944) silver reduction method for glycogen is much more laborious and lengthy, and it has the disadvantage that it is not specific for glycogen, as reticular fibres are heavily impregnated.

SUMMARY

A new method for the histochemical detection of glycogen is described, based on the reduction of silver diamino hydroxide by glycogen after preliminary treatment with chromic acid.

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EXPLANATION OF PLATE

- Fig 1 Section of liver from an adult rat × 150
Fig 2 Section of liver from an adult rat × 600
Fig 3 Section of nasal concha from a 20 day rat embryo × 150
Fig 4 Section of cartilage from a nasal concha of a 20 day rat embryo × 600

THE EFFECT OF PERIPHERAL CONNEXIONS ON THE MATURATION OF REGENERATING NERVE FIBRES

By J T AITKEN, *Anatomy Department, University College, London*

INTRODUCTION

Previous work (Sanders & Young, 1944, 1946; Weiss & Taylor, 1944, Weiss, Edds & Cavanaugh, 1945, Aitken, Sharman & Young, 1947) has shown the importance of the effect which the peripheral connexions have on regenerating nerve fibres. The present work was planned to examine in detail some of the factors which might contribute to this effect.

Answers were sought to the following questions (a) Does the distance travelled by regenerating fibres modify the process of regeneration? (b) Do fibres turn at the cut end of a nerve and grow back in a central direction? (c) To what extent does close contact of regenerating fibres with muscle tissue influence maturation?

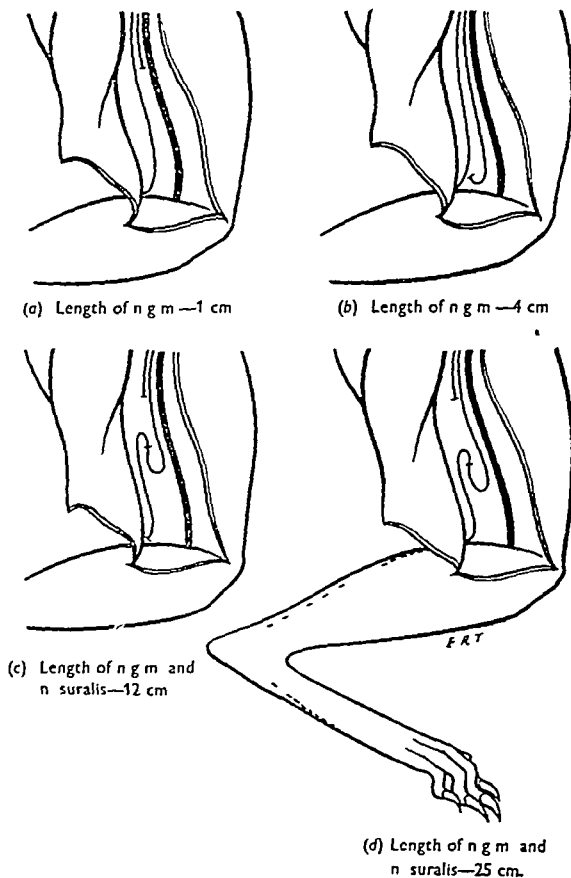
MATERIALS AND METHODS

Adult rabbits of different breeds were used, and the nerve to the medial head of the gastrocnemius muscle (n g m) was selected, as in the previous work (Aitken *et al* 1947). In all cases the n g m was crushed as high as possible, with fine smooth watch-maker's forceps. In order to study the behaviour of new fibres growing down various lengths of nerve but not reaching the end organ, the nerve was also cut distally and the peripheral stump avulsed from the muscle. In this way a neuroma was caused to form at distances 1-4 cm from the crushed point. In order to study regeneration over still longer lengths the proximal cut end of the n g m was joined to the distal end of the severed sural nerve, which lies near by, and in this way the length of the regenerating pathway could be increased up to about 25 cm in large rabbits. The union was maintained by means of coagulated fibrin. In some animals the sural nerve was again cut below the knee (see Text-fig 1).

In this series, regeneration was allowed to proceed for 100 days, and the nerves were then fixed in order to determine the diameter of the new fibres at a level 1.5 cm below the crushed point.

In another series of animals, to determine the effect of the muscle on regeneration, the n g m, after being crushed as high as possible and cut distally, was implanted into a nearby muscle. Biceps femoris was used and the implantation was made near the nutrient artery, by passing the end of n g m along the track in the muscle made by carefully inserting a round-bodied needle between the fasciculi. This manipulation was carried out with the minimum amount of trauma to avoid damaging the muscle fibres. The implant was held in position with coagulated fibrin. The functional state of biceps femoris was varied by removal of the entering nerves from the sciatic trunk on one side, using the opposite side as a control with a normally innervated biceps muscle. In an attempt to prevent reinnervation, the cut ends of the nerve to biceps femoris were formalinized in some animals, but the method was abandoned owing to the danger of injury to the main sciatic trunk.

All animals were examined after a 100-day period and the n g m was identified. In most cases the nerve was stimulated with an induction coil and any muscle response noted. Occasionally, the implantation was not maintained in the muscle and in these cases the nerve formed a neuroma on the surface of biceps or in the fascia.



Text fig 1 Diagram showing the four types of experiment to determine the effect of the distance travelled by regenerating fibres on the maturation of the nerves (a) and (b), lengths of 1 and 4 cm from n g m (c) and (d), lengths of 12 and 25 cm from n g m and n suralis

Where the implant was undisturbed, the minimum stimulus needed to evoke a muscle response was noted with the electrodes placed about 2 cm from the site of the implantation. As far as possible, the stimulation of the nerves was standardized and in each case, after the threshold with the nerve intact had been noted, the nerve was then cut centrally, isolating it from the central nervous system and finally crushed close to the muscle to show whether the contraction was due to spread of the stimulus along the nerve.

In all the different experiments the nerves were fixed in the following modification of Flemming's solution (2% osmic acid in distilled water, 4 cc, 1% chromic acid

in distilled water, 15 c c , glacial acetic acid, 1 drop) for not more than 24 hr , dehydrated without preliminary washing, cleared in cedar-wood oil and embedded in paraffin wax after an intermediate stage in cedar-wood oil-benzol-paraffin wax mixture Sections, 5μ thick, were cut at comparable levels in all nerves In the shortest lengths of n g m the sections were cut as near as possible to the neuroma, but in all others at a point about 1.5 cm below the crush The site of the crush was determined by a slight swelling in the nerve, by the presence of a marker (indian ink or black silk) or by the central cut end of the sural nerve which was found usually close to the crushed point The sections were stained by Weigert's method and photographed at $750\times$ magnification on to bromide paper Differential counts were made of the fibre diameters which were classified into 2μ groups

RESULTS

A *Effect of length of regeneration path on the degree of maturation*

The experiments provided data for the following lengths of the regenerating path (a) regeneration along 1 cm of peripheral path, n g m , (b) regeneration along 4 cm of peripheral path, n g m , (c) regeneration along 12 cm of peripheral path, n g m and n suralis, (d) regeneration along 25 cm of peripheral path, n g m and n suralis

The differential counts of the fibre sizes just below the crush obtained from the nerves in these experiments are given in Table 1 along with the total number of fibres in the nerve, the mean diameter for the fibres over 6μ in diameter and their number

Table 1 (a) shows that in the short peripheral path the total number of fibres is large and usually over 2000 The great majority of these fibres are small and poorly myelinated, and the mean diameter of the groups over 6μ is 7.33μ As the neuroma in these experiments is very near to the crush there will be a large number of fibres which turn at the neuroma and pass back along the nerve They will have been counted twice (see later)

Table 1 (b) contains the results obtained from those nerves where the distance travelled by the regenerating fibres was 4 cm of n g m There is a diminution in the total number of fibres, with an increase in the number of those over 6μ in diameter, the mean of the latter being 7.68μ

Table 1 (c) and (d) show a continuation of this trend, the total number falls with an increase in the over 6μ groups and an increase in the mean diameter to 7.98μ in the 12 cm pathway and 8.74μ in the 25 cm pathway

After an examination of Table 1, it was decided to consider only the larger fibre (over 6μ) groups and the mean fibre diameter of those fibres was taken as an index of maturation in the nerve This was done in an attempt to prevent the number of small fibres (some of which would be counted twice) masking the effect of any change in the number and distribution of the larger fibres From each set of comparable experiments a series of mean diameters was obtained and considered as a sample of nerves regenerating for the given length Is there any simple relationship between the degree of maturation and the length of the regeneration path? Inspection of the figures in Table 1 suggests a high degree of correlation between maturation and length regenerated, but the estimation of the correlation coefficient would only give

the extent of the connexion between the two variables. It would not give any information as to the degree in which change in length affects maturation.

If, however, the mean maturation estimations for all the nerves are plotted against the length regenerated, it appears that there is some reason to anticipate a linear relationship between the two variables. In more statistical terminology we may reasonably expect a significant linear regression of maturation on length of the regeneration pathway. This line can be computed and is shown in the diagram (Text-fig 2), indicating that over the range of the experiments there is an increase

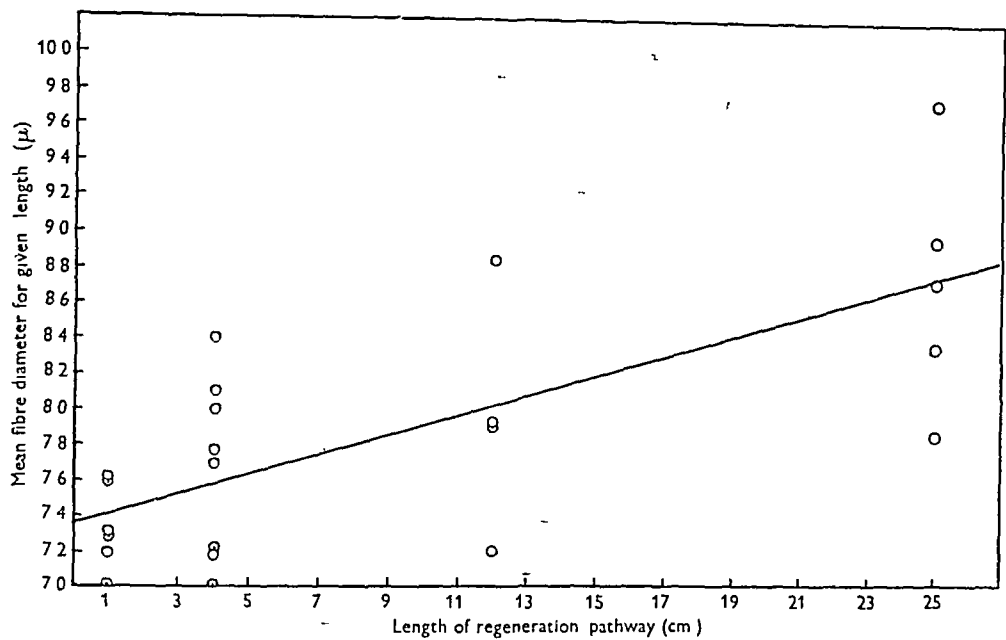
Table 1 *Distribution of fibres in regenerating nerves of different lengths*

Specimen	Diameter (μ)							Total	No >6 μ	Mean of >6 μ fibres	
	0-2	2-4	4-6	6-8	8-10	10-12	12-14				14-16
(a) N g m with 1 cm peripheral path											
48 (a)	326	1254	381	91	8	—	—	—	2060	99	7.32
51 (f)	96	1528	558	207	66	8	—	—	2463	281	7.58
56 (f)	311	2513	521	198	81	2	—	—	3625	281	7.60
57 (f)	378	1576	409	199	22	—	—	—	2584	221	7.20
58 (c)	216	1115	363	150	27	—	—	—	1871	177	7.31
59 (a)	315	1750	375	23	—	—	—	—	2463	23	7.00
Mean of group										7.33	
(b) N g m with 4 cm peripheral path											
29 (b)	132	724	318	124	41	9	3	—	1351	177	7.77
51 (a)	95	764	349	102	10	—	—	—	1320	112	7.18
55 (a)	41	540	193	125	115	41	—	—	1055	281	8.40
56 (a)	35	560	166	162	107	18	—	—	1048	287	8.00
57 (c)	85	711	311	122	3	—	—	—	1222	125	7.05
58 (a)	186	1058	368	128	16	—	—	—	1756	144	7.22
59 (b)	45	583	194	124	117	11	—	—	1074	252	8.10
185 (b)	36	518	263	138	67	3	—	—	1025	208	7.70
Mean of group										7.68	
(c) N g m joined to sural nerve—total length 12 cm											
83 (a)	35	414	172	112	160	73	6	—	972	351	8.85
89 (a)	53	589	279	119	68	11	—	—	1199	198	7.91
95 (a)	61	503	117	143	110	5	—	—	939	258	7.93
105 (b)	124	322	270	233	5	9	1	—	964	248	7.21
Mean of group										7.98	
(d) N g m joined to sural nerve—total length 25 cm											
89 (c)	20	589	277	144	114	46	—	—	1190	304	8.36
539 (d)	55	274	108	94	120	58	—	—	709	272	8.74
539 (a)	14	160	110	119	129	104	5	—	641	357	8.97
541 (a)	56	444	225	152	93	8	—	—	978	253	7.86
546 (a)	23	192	83	86	106	94	53	5	642	344	9.75
Mean of group										8.74	

of 0.5 μ in the mean diameter of the larger regenerating fibres in each 10 cm travelled. That the effect is a real one and not due to chance is shown by the regression coefficient which is significant on the 0.001 level ($t=4.7$, $f=21$).

These experiments therefore definitely indicate that regenerating fibres which grow for a greater distance become larger. Nothing very definite can be said about the form of the relationship, the diameter more nearly follows the length than its logarithm, but other possibilities are not excluded. It must also be remembered that the very longest fibres make endings different from the others (in the skin rather than in a neuroma).

As the length of the peripheral path and the rate of progress of the nerve fibres down the path can be estimated, it is possible to calculate the time taken to reach the end of the Schwann tubes, and by deducting this figure from 100 to determine the time allowed for maturation. Allowing 5 days for the fibres to cross the crush, 5 days to cross the union and a growth rate of 5 mm per day above the union but of only 3 mm a day below the union (Gutmann, Guttman, Medawar & Young, 1942) we obtain the results summarized in Table 2



Text fig 2 Linear regression of degree of maturation on the length travelled by the regenerating nerves maturation being measured as the mean diameter of the larger ($> 6\mu$ in diameter) fibres

Table 2 The estimated times allowed for maturation of the regenerating fibres of n g m after travelling different distances

Distance travelled (mm) .	Estimated time to reach end of nerve (days)	Time allowed for maturation (days)
10	7	93
40	13	87
120	45	55
250	88	12

In the longest nerves many of the fibres would be actively growing until 12 days before the biopsy. The specimens with neuromas at the cut end of the n suralis below the knee (12 cm) would have 55 days in which to mature, yet the mean diameter of the fibres is less than that found in the longest nerves (25 cm) (see Text-fig 1)

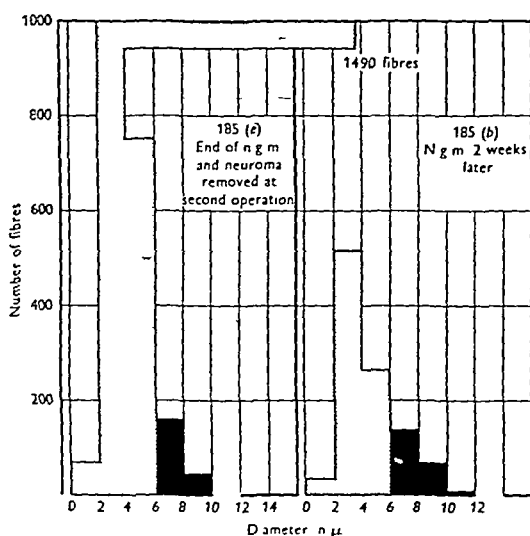
B Turning back of regenerating fibres at a neuroma

When regenerating nerve fibres reach the end of the Schwann tubes, without making contact with a peripheral end organ, a neuroma is formed. It has been suggested by Weiss *et al* (1945) that some of the fibres turn and re-enter the Schwann tubes and travel in a central direction. This phenomenon was clearly demonstrated

in one of the experiments where the nerve was crushed, cut lower down, allowed to regenerate and form a neuroma, and was then operated on again, the neuroma being cut off 2 weeks before the terminal biopsy. Comparison of the two specimens 185 (e) and 185 (b) in Table 3 shows that the total number of fibres had been reduced to one-half

Table 3 (a) and (b) Counts of regenerating fibres in the n g m immediately proximal to a neuroma and in the same nerve 2 weeks after the neuroma had been removed

Specimen	Diameter (μ)						Total
	0-2	2-4	4-6	6-8	8-10	10-12	
(a) Portion of n g m with neuroma attached							
185 (e)	68	1490	750	158	42	—	2508
(b) N g m 2 weeks after removal of neuroma							
185 (b)	36	518	263	138	67	3	1025



Text-fig 3 Histograms of the distributions of fibre sizes in n g m immediately proximal to a neuroma and in the same nerve two weeks after the neuroma had been removed

Unfortunately, owing to the nearness to the neuroma, it was impossible to obtain a section of which all parts could be counted. However, a reliable estimate was made by determining the total area of the photograph with a planimeter (727 sq cm) and calculating the number of fibres in the size groups from the area which was capable of being counted (426 sq cm).

Examination of the figures in Table 3 shows that the greater differences are to be found in the groups of smaller fibres. From these findings it does seem that a great number of small regenerating fibres returns along the nerve trunk and thus suggests that the larger fibres are the better index of maturation as the number of larger fibres was almost the same in the two specimens.

Text-fig 3 shows the histograms of these two specimens, and Pl 1, figs 1 and 2 the photographs of the cross-sections which were counted. The difference in size of the sections is due to the proximity to the neuroma and the number of fibres

C Effect of muscle state on maturation of regenerating fibres

On examination of the animals after 100 days, it was found that the biceps muscle was not greatly wasted on the paralysed side, though stimulation of the sciatic nerve high in the thigh produced no contraction of the muscle. Stimulation of n g m gave rise to a contraction of a bundle of muscle fibres. By increasing the strength of the stimulus it was possible to make more muscle fibres respond, though never did the muscle react as when the nerve to the normal biceps on the other side was stimulated. The paralysed muscles responded at a higher threshold of stimulation of the nerve than did the normal muscles. The differential counts of the fibre populations of these nerves are given in Table 4.

Table 4 *Distribution of fibres in regenerating n g m when implanted into normal and paralysed biceps femoris muscles*

Specimen	Diameter (μ)							Total	No >6 μ	Root mean sq diameter of fibres >6 μ (D)
	0-2	2-4	4-6	6-8	8-10	10-12	12-14			
(a) Implantation into normal biceps										
417 (<i>r</i>)	52	1035	256	177	23	—	—	1790	200	7.2
428 (<i>r</i>)	115	716	287	165	67	8	—	1358	240	7.9
443 (<i>c</i>)	175	586	194	163	81	—	—	1199	244	7.7
483 (<i>a</i>)	316	973	419	77	216	2	—	2003	295	6.3
642 (<i>c</i>)	12	588	375	228	66	10	—	1279	304	7.4
643 (<i>b</i>)	55	426	193	189	78	1	2	944	270	7.7
(b) Implantation into paralysed biceps										
417 (<i>l</i>)	6	817	394	326	160	97	11	1811	594	8.5
428 (<i>l</i>)	85	730	285	150	29	13	—	1292	192	7.7
443 (<i>a</i>)	151	612	183	134	66	9	—	1155	209	7.9
483 (<i>d</i>)	138	968	185	85	111	69	49	1605	314	9.7
642 (<i>a</i>)	13	347	133	110	105	65	30	803	310	9.3
643 (<i>c</i>)	44	416	216	136	98	36	5	951	275	8.5

The larger fibres (>6 μ) were again used as an indication of the degree of maturation. The hypothesis that the distributions of the larger-sized fibres are the same whether the nerve terminates in a normal or paralysed muscle was made and calculations gave the following results:

Animal	X^2	f	$P(X)^2$
417	50.84	1	0.001
428	11.757	2	0.01
443	0.36	1	0.6
483	13.91	2	0.001
642	118.86	2	0.0001
643	21.8	2	0.001

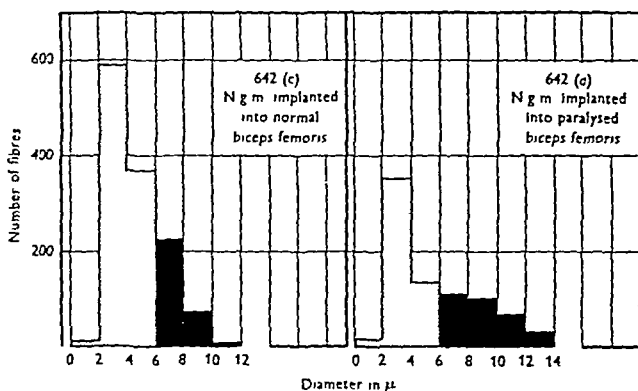
Except in animal 443, the hypothesis is rejected in every case. When the total X^2 is considered, $X^2=217.53$, $f=10$ and $P(X)^2=0.001$.

The possibility of these samples belonging to the same population is therefore exceedingly remote.

When the root mean square diameters (D) of the fibres more than 6 μ in diameter

are considered it is evident that the nerves which regenerate into the paralysed muscles are larger in all experiments except one (428)

Pl 1, figs 3 and 4 show photographs of sections of nerves (642*a* and 642*c*) which have been implanted into normal and paralysed muscles. It will be noticed that the former produces a condition in which the Schwann tubes are filled with many small fibres whereas, in the latter, the tubes contain fewer fibres and usually one large fibre. Histograms of the same two nerves (Text-fig 4) demonstrate the reduction in the total number of fibres—especially the smaller groups—and an increase in the larger fibre groups.



Text fig 4 Histograms of the distributions of fibre sizes in regenerating *ngm* after implantation into normal or paralysed *biceps femoris*

DISCUSSION

From the preceding results, it is evident that the distance travelled by regenerating nerve fibres and the possibility of making a functional connexion with muscle both influence in a marked degree the process of maturation.

A Effect of length

The diameters of normal nerve fibres are usually taken to vary according to the type of functional activity of the fibres (Erlanger & Gasser, 1937). In these experiments, the regenerating fibres rarely attain a size more than 12–14 μ in diameter, they are non-functional and therefore the peripheral connexion can have no stimulating effect on the maturation processes. Where the *ngm* was united with the *n. suralis*, certain new factors were introduced which must be considered. The union of the nerves, the smaller Schwann tubes of the sensory nerve and the presence of peripheral end-organs in the skin are the most important factors. The unions were made with no tension between the two nerves and the positions were maintained with coagulated plasma. At biopsy, the unions were examined and found to be intact and sections of the *n. suralis* proved that many fibres had passed down into this nerve. Pl 1, fig 5 is a photograph of a longitudinal section of one of these unions (95*d*). There is always the possibility, however, that a few fibres escaped at the site of the union into the surrounding fascia or even turned back up the *ngm*.

The smaller Schwann tubes might tend to impede the flow of axoplasm and so increase the diameter of the fibres above the union

In the normal n g m there are a number of sensory fibres which might find a pathway towards an end organ in the skin. These fibres were presumably non-functional, as stimulation of the skin supplied by n suralis evoked no response, and when the n suralis was electrically stimulated no withdrawal of the leg occurred.

The effect of the length of nerve regenerated was investigated by Sanders & Young (1946), who considered that the process of maturation was independent of it. They employed varying lengths of the large peroneal nerve and joined it to the still larger tibial nerve. Both of these nerves have considerable mixed muscle and sensory (skin) components and the regenerating fibres would eventually make contact with end organs, many of them on the muscle fibres. Though the two sides would be partly comparable, no indication is given of the possibility of the motor end-plate connexion masking the length effect. The difference in length was 55 mm, which according to the present findings would account for a shift upwards of 0.25μ in the mean diameter of the fibres, a figure which could almost certainly be accounted for by the varying peripheral connexions of the fibres.

Time for maturation It has been suggested by Weiss *et al* (1945) that the nerve fibre increases in diameter after the growing end comes to rest. In the present series of experiments, those nerves which could regenerate along the terminal branches of n suralis into the foot would have only 10–20 days in which to mature, whereas those nerves which ended in neuromas in the thigh or leg would have 70 or 50 days. The degree of maturation, however, bore an inverse relationship to the time. In spite of the long time which could be used for maturation, the shorter lengths had nerves of a smaller mean diameter than the longer nerves. Previous work (Aitken *et al* 1947) has shown that when nerves are allowed to regenerate and form neuromas for periods up to 200 days, though the number of fibres is reduced in the longer periods, there is no increase in the diameter of the fibres. It is therefore probable that in considering the degree of maturation the distance travelled is a more important factor than the time.

B Effect of overcrowding

The effect of overcrowding the Schwann tubes by a large number of small fibres is most pronounced in the shorter lengths of nerve. Their numbers over the total series of nerves vary considerably and for no very obvious reason, even some of the implants into paralysed muscle (Table 4 (b)) having a total of over 1000 fibres in the groups under 6μ in diameter. The larger-sized fibres seem to form a more constant and stable series, and in the nerve which had the neuroma removed 2 weeks before biopsy there was a difference of only 8 between the number of large fibres in the specimen of nerve with the neuroma and that found in the nerve itself. It is realized that there is a difficulty in making a definite statement concerning this matter as sections cut near the neuroma are very difficult to count accurately owing to the obliquity of many of the fibres.

When Pl 1, figs 1 and 2 are compared, it is seen that many of the Schwann tubes in fig 2 (section of n g m after removal of the neuroma) contain only one fibre and often this fibre is small. During the 14 days since operation, many of the small fibres have degenerated, but the remainder do not seem to have increased in size.

C *Effect of muscle state*

It has long been maintained that the nerve supply of mammalian muscle is such that each muscle fibre has one motor end-plate (Wilkinson, 1929, Denny-Brown & Pennybacker, 1938), though some workers (Aghduhr, 1916, Cuajunca, 1932) have reported the presence of multiple endings on a fibre. In normally innervated muscle there will therefore be a state of equilibrium between muscle and nerve, and when a 'foreign' regenerating nerve is made to grow into a fully innervated muscle, it will lie in contact with muscle fibres which are already innervated. Yet when the nerve is examined the process of maturation is found to have proceeded farther than in a nerve of comparable length which forms a neuroma in the fascia (compare results in Tables 1 (b) and 4 (a)). The close proximity to muscle tissues does thus seem to have an effect on the process of maturation. Examination of nerves in normal muscles which is being carried out shows that many fine fibres pass between the muscle fibres with little or no attempt to form motor end-plates.

When a regenerating nerve is implanted into a paralysed muscle the process of maturation is greatly facilitated (Table 4 (b)), not only is the number of small fibres usually reduced but the larger fibres are significantly increased. When these results are compared with those previously reported (Aitken *et al.* 1947) for maturation in regenerating nerves following union with a muscle nerve, it is found that the maturation after implantation is poorer than after union. In the latter case, most of the motor end-plates were reinnervated and the whole muscle contracted on stimulation. Stimulation of an implanted nerve in a paralysed muscle gave rise to contraction of a few bundles of muscle fibres. Histological examination of the paralysed muscle revealed no innervated 'native' motor end-plate but a very different neuroma from that which was found in a normal muscle. It would seem that the implant responds to the demand on the part of the paralysed muscle and that the most potent stimulus to maturation is the opportunity to form new motor end-plates. As counts of the new motor end-plates were not made, it is impossible to correlate the degree of maturation with the number of functioning muscle units but the number of endings was never very great and they were mostly close to the implant.

The growing ends of the nerve fibres would reach the muscle in about 13 days after the operation and they would have 87 days in which to ramify amongst the muscle fibres. The results of electrical stimulation showed that the functional spread of the nerves was restricted. This was specially marked in the experiments where the implantation was made into a normal muscle.

Fort (1938), working with Weiss, has studied some of the factors involved in the establishment of neuro-muscular connexions in toads. He suggests that denervated muscle may be more 'permeable', though he admits that his evidence is inconclusive. Attempts to arrest the process of reinnervation in a denervated muscle by use of a Ringer extract of normal muscle were also not effective.

From the present work it would seem that the juxtaposition of regenerating nerve fibres and muscle fibres is sufficient to initiate the process of maturation, but that the opportunity of forming a functional motor end-plate on muscle fibres is a very strong stimulus to continuation of the process.

SUMMARY

1 Experiments in which nerves were allowed to regenerate along pathways of different lengths (1, 4, 12 cm ending in a neuroma, and 25 cm ending in the skin) showed that the longest nerves had largest diameters. Over the range investigated there was an increase of 0.5μ in the mean diameter of the fibres for each 10 cm travelled.

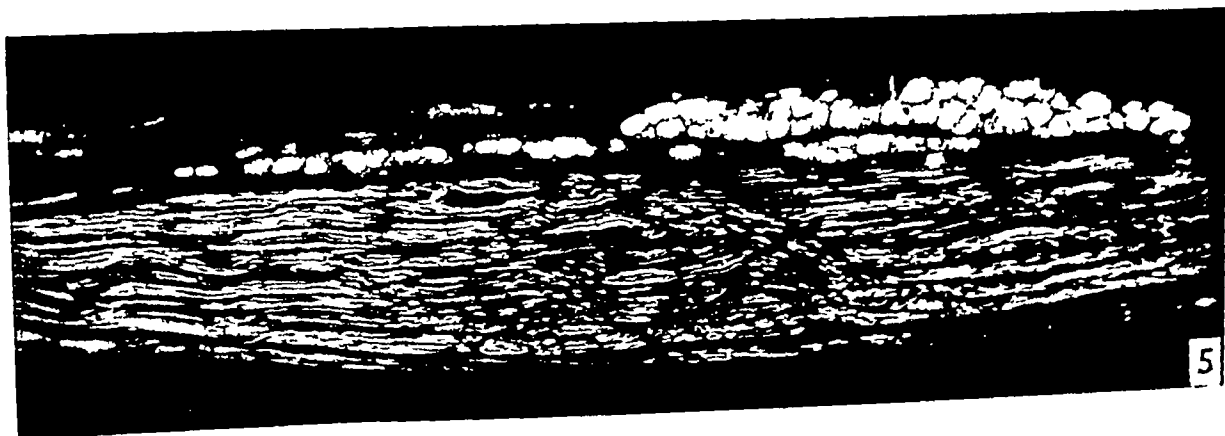
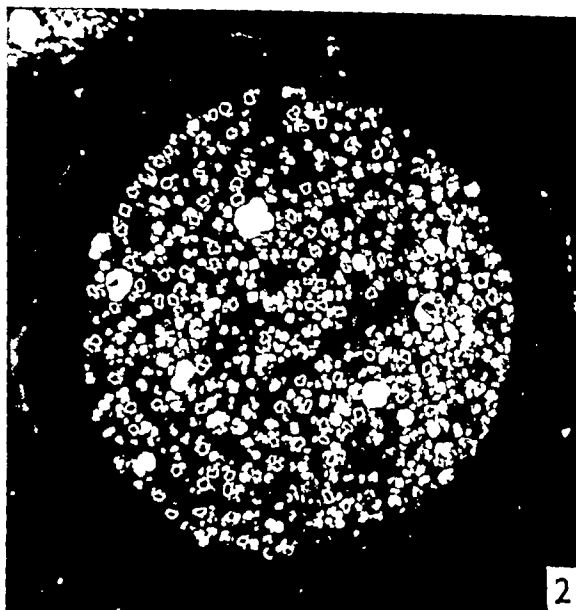
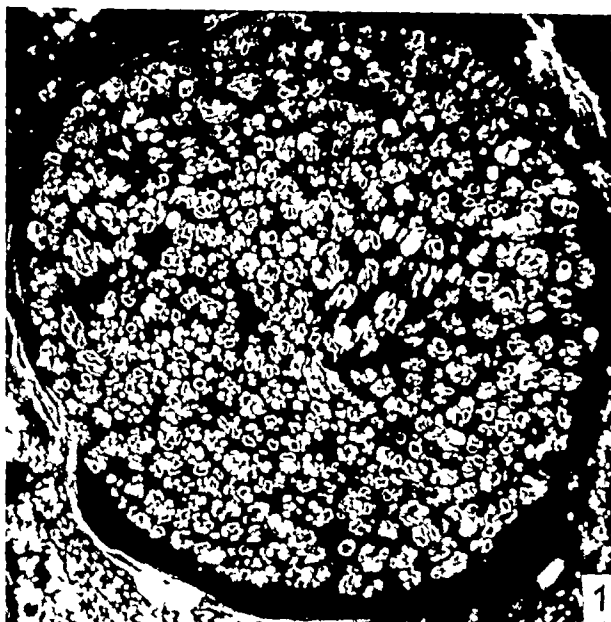
2 Almost half the regenerating fibres turn round at a neuroma and travel back along the nerve. This effect is greatest in those cases where the regeneration path is short.

3 Maturation of a regenerating nerve is much more complete when the nerve fibres are allowed to make contact with paralysed (denervated) muscle fibres than when the nerves grow into a normal muscle or into fascia. The possibility of making new functional motor end-plates or reinnervating those which have been denervated produces a marked increase in the degree of maturation.

The author wishes to thank Prof J Z Young for his interest and encouragement with this work, Mr D Sholl for the statistical analyses, Mr L Hanning for technical assistance.

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EXPLANATION OF PLATE

All figures are of specimens fixed in Flemming's solution, Weigert-stained and photographed directly on to bromide paper

Fig 1 Regenerating nervus gastrocnemii medialis immediately proximal to the neuroma (185e) ($\times 150$)

Fig 2 Regenerating nervus gastrocnemii medialis 2 weeks after removal of the neuroma (185b) ($\times 150$)

Fig 3 Regenerating nervus gastrocnemii medialis which was implanted into a normally innervated muscle (642c) ($\times 150$) ,

Fig 4 Regenerating nervus gastrocnemii medialis which was implanted into a paralysed (denervated) muscle (642a) ($\times 150$)

Fig 5 Longitudinal section of union between nervus gastrocnemii medialis and n. suralis 95d) ($\times 55$)

REVIEWS

Gynaecological and Obstetrical Anatomy By C F V SMOUT, M D M R C S and F JACOBY, M D , Ph D 2nd ed (Demy 8vo Pp x1+248 With 185 illustrations 40s) London Arnold

The present book is in effect a second edition of *The Anatomy of the Female Pelvis* first published in 1943

The book is divided into nineteen chapters and gives an account of the anatomy of the pelvis, the development and histology of the female genital tract, endocrine function of the ovarian hormones, the placenta, and the anatomy of the foetus in relation to childbirth

The chapters describing the anatomy of the pelvis are concise and accurate, but not quite as comprehensive as one would have wished to have in such a volume For example, no reference is made to the masterly contribution by Dickinson on 'Human Sex Anatomy' Some of the rather elaborate diagrams are too schematic For example, Fig 96 shows the vagina as a straight tubular structure, whereas in fact it should be shown as curved in the sagittal plane, as in Fig 97

The chapters dealing with the histology of the ovary and with ovarian function and its control give a valuable account of the advances that have been made in these fields during the past decade As this book is concerned mainly with human anatomy and histology it would have been more appropriate to have had more illustrations of the human ovary than of the cat

The book has been accepted as a standard text-book and it is likely to have a long run with successions of editions For this reason the reviewer has suggested the above comments for consideration by the authors The book will be of value to those who are desirous of having a concise account of the anatomy and physiology of the female pelvis in one volume

W J HAMILTON

Chronic Structural Low-backache due to Low-back Structural Derangement By R A ROBERTS, B Sc , M B , Ch B , D M R E (Pp 105 (exclusive of 45 plates with 137 illustrations), 1947, 45s) London H K Lewis and Co , Ltd

This is a valuable book for all concerned with differential diagnosis in patients with low-backache Sixty-four case-histories are presented, the great majority of which show defects in the vertebral neural-arch, duly illustrated radiologically The term *pars interarticularis* or isthmus of the neural-arch is defined as the narrowest and most delicate part of the neural-arch in the lumbar spine situated between the superior and inferior articular processes The author considers this isthmus the vital pivotal centre in the architecture of the vertebrae for the interaction of the powerful muscles and ligaments of this region, because first, in the antero-posterior plane it is the pivotal centre between the pedicle with the body, and the lamina with the spinous process, secondly, in the vertical plane it lies between the superior and inferior articular processes which are most concerned with guiding the vertebral movements, and thirdly, in the lateral horizontal plane it is at the base of the transverse process

The literature of separate neural-arch is discussed Although absent in the foetus, its incidence, generally accepted at 5 %, has been found up to 10 % and even 25 % There must be many individuals with separate neural-arches in the last lumbar vertebrae who have never suffered low-backache Dr Roberts submits the view that these defects are primarily the result of overstrain de-ossification, and are indeed akin to 'march' fracture or 'stress' fracture In view of the fact that at least one highly competent radiologist has previously reiterated that such defects could not be demonstrated by X-ray, Dr Roberts is

to be congratulated upon producing the radiological evidence of these bilateral and unilateral defects of the pars interarticularis. He insists that oblique lateral views at about 30° from the antero posterior position are essential for their demonstration. These deformities in themselves are not the cause of all the symptoms presented by the patients, but they indicate a structural defect, a flail joint, which must be counteracted by the soft tissues which are more susceptible to overstrain. He concludes that trauma is only an incidental aggravating factor in these cases, two thirds of the cases did not reach their early twenties without suffering pain in the back. The oedema arising from overstrained soft tissues with consequent nerve pressure, is held responsible for the pain. In discussing neuro-vascular and visceral symptoms presented by patients, the author states that 'skeletal overstrain can affect the nearby sympathetic nerves causing anterior and visceral neuro vascular disturbance'.

The histories of the patients have been carefully elicited, and the tragedies recounted of men of athletic activity and patriotic spirit, who have suffered the stigma of malingerer, neurotic and even mental defective, are well calculated to cause the clinician to ponder gravely and pursue his case history and technical investigation exhaustively, before committing himself to diagnosis in these difficult cases.

We may sympathize with the author's question 'Since most of the present authoritative writings by gynaecologists, urologists, abdominal surgeons, "internists" and psychiatrists pay little attention either to the pain of spinal origin which may simulate visceral pain, or to the neuro vascular symptoms which can arise through sympathetic irritation by skeletal lesions, is there not a need for a radical reassessment of our differential diagnoses?'

Dr Roberts agrees with many of his predecessors that these troubles are ultimately due to man's adoption of the erect posture. Surely this theme has been long overdone. The more we study anatomy the more we marvel at the excellence of Nature's handiwork, but certainly it is a surprise that more slips do not occur during the embryonic adventures of any animal in attaining the standard pattern. It may be that many anatomists and clinicians will consider that the author sometimes overstretches his thesis, but that is to be expected in the introduction of a doctrine.

Examination of the radiographs clinches the argument in favour of those who maintain that their interpretation is strictly a matter for the expert radiologist. Indeed, as a side issue to the teacher of anatomy, it is a debatable point whether the substitution of his students of the shadow for the substance, has not already gone beyond reasonable limits.

A minor point of criticism—possibly due to the case-histories having been culled from field records—is that the average reader is probably still pedantic enough to object to such abbreviations as 'Pain wakes him at night, gets up and walks about for a while'.

The format of the publication is excellent.

R D LOCKHART

Cardiovascular Studies By KENNETH J FRANKLIN, D M, F R C P (1948 Pp xvi + 306 With frontispiece and 102 figures) Oxford Blackwell Scientific Publications

This is an interesting work. It is written with the scholarship and erudition which mark all Prof Franklin's writings, though we think he is at his best in Part I, a historical 'note' running to 47 pages, on the Eustachian valve and the intervenous tubercle of Lower. Apart from the smoothly flowing and felicitously worded narrative, the author brings to this historical study an enthusiasm which is contagious, and which whets our appetite for the rest of the book. Part II has not quite the unity and cohesion of Part I, for it consists of miscellaneous elements, including a brief discussion of terminology, a description of techniques, an account of how the essential ideas underlying the book developed, references to the literature, and observations on the human heart. On the question of terminology, we must confess to some unhappiness over the author's substitution of 'crusta interveniens' for the intervenous tubercle.

Part III constitutes the main bulk of the book, and consists of references to the literature and detailed observations on a large number of mammalian hearts. This part cannot be read in the ordinary sense of the word, but it is an exceedingly valuable compilation of factual material for reference purposes. Apart from the great mass of descriptive matter in Part III, matter which will assuredly be of permanent value irrespective of the author's interpretation of it, his main concern is with the significance and formation of the Eustachian valve. For this purpose he introduces two novel criteria for classifying hearts, namely the Heart Type, and the Heart Index.

The Heart Type is determined by the direction of blood flow in the superior vena cava, which on entering the heart may impinge (a) on the caudal floor of the right atrium immediately to the atrial side of the Eustachian valve, the medial cornu of which is attached either to the surface of the fossa ovalis dorsal to the sternal pillar of its limbus [type I], or to the sternal pillar [type II], (b) on the caudal floor of the right atrium between the Eustachian valve and the right atrio-ventricular orifice [type III], (c) directly into the right atrio-ventricular orifice [type IV]. The Heart Index is the

$$\frac{\text{maximum height of Eustachian valve}}{\text{heart length}} \times 100,$$

and indicates the degree of development of the Eustachian valve, it should perhaps be more aptly designated the 'Eustachian valve index'. The main thesis of the book is that there is a correlation between Heart Type and Heart Index, and that furthermore the formation of the valve is stimulated haemodynamically, its height depending upon the angle of impingement on the atrial floor of the blood of the superior vena cava. This is an interesting concept, though one which it is difficult to submit to experimental verification. However, we agree with the author that the validity or otherwise of this concept will not 'reduce the general value of the book as a contribution to the comparative anatomy of the mammalian heart, and as an introduction to the literature on that and on allied subjects'.

Any review of this book would be incomplete if it failed to draw attention to the fact that the author, a Professor of Physiology, has contributed a study which at first sight appears to follow the tradition of classical morphology. Throughout the book, however, he is writing against the background of the experimental studies so brilliantly conducted by himself and others, and is thus enabled to arrive at a firmly grounded functional evaluation of his observations. Prof. Franklin's work represents an admirable illustration of harmonious combination in the study of structure and function. There are anatomists to whom function merely spells a series of airy generalizations and speculations based on scanty morphological observations. Not so the author of this book. By his assiduous devotion to morphology on the one hand, and the avidity with which he employs the most effective experimental techniques available on the other, Prof. Franklin has shown himself to be a worthy modern exponent of the true Hunterian tradition.

J. M. YORRY



JOHN KAY JAMIESON

From the portrait in the Leeds University School of
Medicine, by Mr Leo Whelan, Dublin, 1937

IN MEMORIAM

JOHN KAY JAMIESON, M B , C M (Edin), LL D (Leeds),
M A (Dublin), M CH (Dublin), 1873-1948

John Kay Jamieson, Professor of Anatomy in the University of Leeds for twenty-six years and in the University of Dublin for another eleven, died at Dublin on 20 August 1948, aged 75 By his death the Anatomical Society loses one of its oldest members, as he was elected at the famous Summer Meeting of 1897, in Trinity College, Dublin

Born in Shetland in 1873, he was the fourth son of Robert Jamieson of Sandness, other members of whose family also distinguished themselves academically He attended the Sandness Madras School which his father had established before the Scottish Education Act of 1872 with the assistance, it is understood, of the trustees of the Rev Dr Andrew Bell (1753-1832), himself the founder of the Madras College at St Andrews Jamieson's eldest brother, Francis, after a period as Lecturer in Humanity in the University of Edinburgh, became H M Chief Inspector of Schools for Scotland, his two younger brothers entered the medical profession—Dr E B Jamieson, until lately Senior Lecturer in Anatomy, University of Edinburgh, and Dr J P S Jamieson of Nelson, New Zealand

In 1894, after graduating M B , C M at the University of Edinburgh, Jamieson took up his first post as Demonstrator of Anatomy with Macdonald Brown, Lecturer in the Extra-mural School of Medicine, at Surgeons' Hall Next year he was appointed Demonstrator under Wardrop Griffith at Leeds, and so began a period of 41 years' service to the School of Medicine there In 1910, when Griffith was translated to the Chair of Medicine, Jamieson was the natural choice to succeed him as the first whole-time Professor of Anatomy in the University of Leeds

From 1910 to 1914 he served as Sub-Dean of the Faculty of Medicine and was Resident Warden of Lyddon Hall, the hostel for men students But it was during the 1914-18 War that his talents as administrator led to serious demands on his time and energy which were thus heavily mortgaged to the conduct of affairs throughout the rest of his period in Leeds From 1914 to 1918 he acted as Dean of the Faculty of Medicine and was Registrar, later Commanding Officer with the rank of Lieut - Colonel, of the East Leeds War Hospital In 1918 he was formally appointed Dean of the Faculty, an office which he retained until he left Leeds in 1936 During these eighteen years he was continuously a member of the University Council, he was Pro-Vice-Chancellor 1923-25 and in 1923-24 Acting Vice-Chancellor of the University From 1928 to 1936 he also represented the University of Leeds on the General Medical Council, and it is not surprising that he came to be regarded as the dominant personality in the Leeds School of Medicine

Jamieson's outstanding services to the University of Leeds and to its School of Medicine in particular have indeed been handsomely acknowledged by one of his colleagues (*Lancet*, 18 September 1948, p 479, and *Leeds University Review*, 1948) Here it is sufficient to record that he was the chief architect of the great development of the Leeds School of Medicine during the inter-war years—the establishment of

a new School of Dentistry in close relation to the General Infirmary (1928), the extension of the Department of Physiology (1930), with subsequent provision of Departments and Chairs of Biochemistry and Pharmacology, and the building of the Algernon Firth Institute of Pathology (1933). On the occasion of his departure from Leeds for Dublin in 1936, the University of Leeds also acknowledged its debt to Jamieson by conferring on him the degree of LL.D. and by the presentation of a characteristic portrait that now hangs in the Library of the School of Medicine.

For in 1936, to the surprise and regret of all his colleagues, Jamieson decided to accept an invitation from the University of Dublin to succeed A. Francis Dixon in the combined chairs of Anatomy and Surgery and of Human Anatomy and Embryology. A transfer from one University to another after 41 years' service is unusual, but Jamieson was then approaching the normal age of retirement at Leeds, and Trinity College offered him an additional five years' service to Anatomy unburdened by excessive administrative demands. In the event, owing to the outbreak of war and other factors, he remained in office at Trinity College for eleven years until his final retirement in December 1947. The University of Dublin admitted him to the degrees of M.A. in 1938 and of M.Ch. in 1947, the latter degree an unusual distinction indicating the high value set on his work by his surgical colleagues.

Jamieson was no stranger to Trinity College and Dublin, he was welcomed there as a friend, and he quickly made an honoured place for himself in academic and medical circles and in the affection of his students and colleagues (*Lancet*, 4 September 1948, p. 397). He was at once elected a Fellow of the Royal Academy of Medicine in Ireland, and he served later as President of the Section of Anatomy and Physiology. He took a great interest in the well-being of that Section—his usual pithy comments became a feature of its discussions—and he contributed two papers to its *Proceedings*: 'The distribution of lymphatics' (1936-37) and 'The teaching of Anatomy' (1941-42). In relation to his older Chair of Anatomy and Surgery, which originally carried beds in Sir Patrick Dun's Hospital in Dublin, he was appointed Consulting Anatomist to that hospital, and he was elected also a Member of the Royal Irish Academy (1937).

As a teacher of Anatomy Jamieson's reputation was early established, and there is abundant testimony that it stands very high with his former students at both Leeds and Dublin. He was a regular attender at the Leeds and West Riding Medico-Chirurgical Society, of which he was made an honorary life-member—a rare distinction, and his teaching was strongly influenced by practical clinical considerations in the Leeds tradition. His lectures on Anatomy in relation to function and to practice, delivered in a very characteristic style and abundantly illustrated by simple diagrams, held the attention of his students and made them think for themselves. He excelled in the intimate teaching of the dissecting room, and he took great delight in the demonstrations that he continued to give there during the whole period of his professorships.

As an examiner Jamieson had a very wide experience and was adept in assessing a candidate's knowledge in a short space of time. He had examined in most of the British Universities, and during the 1914-18 War, and for some time after, he served continuously as External Examiner at Trinity College so that, as has been said, he was no stranger when he arrived in Dublin in 1936. At one period during the Second World War, owing to difficulties of travel, he

enjoyed, and confessed that he enjoyed, the unique distinction of examining in *all* the Schools in Ireland

Jamieson's well-known contributions to the Surgical Anatomy of the Lymphatic System—made principally before he was immersed in administrative duties—have stood the test of time. The reputation of Leeds in Surgery and the fact that a number of rising young surgeons, including Berkeley Moynihan, were Honorary Demonstrators of Anatomy and among his first friends in Leeds, directed his interest to investigations that would assist in surgical advance. With the late Mr J F Dobson he was early engaged in the time consuming labour of investigating the lymph-drainage of various regions and organs by means of Gerota's method, of which they published a brief account 'On the injection of lymphatics by Prussian blue' (*J Anat* 1911, 45, 7). The main fruit of these labours was a series of papers that began to appear in 1907, first as the Arris and Gale Lectures on 'The lymphatic system of the stomach' (*Lancet*, 1907, 1, 1061) and 'The lymphatic system of the caecum and appendix' (*ibid* 1907, 1, 1137), followed by 'Excision of the caecum and ascending colon with the corresponding lymphatic area' (*ibid* 1908, 1, 149), 'The lymphatics of the colon' (*Proc Roy Soc Med* 1909, 2, 149), in which their well-known grouping of the colic lymph-glands was described, and 'The lymphatics of the testicle' (*Lancet*, 1910, 1, 493). At the Summer Meeting of the Anatomical Society in July 1907 at Edinburgh, they had given a 'Demonstration of lymphatic vessels and glands injected according to the method of Gerota' (*J Anat* 1909, 43, *Proc* p 11) and, after the interruption of the 1914–18 War, the last paper in this notable series appeared 'The lymphatics of the tongue with particular reference to the removal of lymphatic glands in cancer of the tongue' (*Brit J Surg* 1920, 8, 80). In that paper they named the jugulo omohyoid gland as a companion to the jugulo-digastric gland of Leaf—limiting between them the deep cervical glands that receive lymph directly from the tongue—and they said the last word about the lymph-drainage of that organ while operative excision was still the treatment of first choice.

Although radio-therapy may tend to replace operative surgery in the treatment of malignant disease, these studies of Jamieson and Dobson in the Surgical Anatomy of lymphatic drainage retain their basic value, and the usefulness of their illustrations to students is testified by their inclusion in our standard text-books.

Jamieson, like many other anatomists, was an open-air man in such spare time as he had. He was an ardent golfer—and we use the adjective advisedly. His walk was always characteristically rapid and, so far as the field in front of him would allow, he raced round a golf course as if there were no time to lose, his partners and opponents sometimes hard put to it to keep up with him. He was a regular player at the Alwoodley Club in Leeds and he joined the Island Golf Club at Dublin. He took part in all the Staff Competitions at Trinity College and won the Staff Golf Cup twice. It was one of his chief regrets, though he made light of it, that increasing disabilities in later years prevented him enjoying the strenuous exercise on the golf course to which he had been accustomed.

Of Jamieson the man, a personal appreciation by one of us has already appeared (*Lancet*, 4 September 1948, p 398), and a striking testimony to his popularity among his students and the esteem in which they held him has been contributed by one of themselves (*Brit med J* 11 September 1948, p 535). With his Norse origin, he was

a man of arresting personality that instantly impressed all with whom he came in contact. On further acquaintance, as his rugged appearance and his unconventional outlook and manners became familiar, few could resist the kindly disposition and the essential friendliness that they imperfectly concealed. Well read in classical literature, he was steeped in his native lore, and he had a fund of curious information on surprising subjects. His conversation was not only always stimulating but also on occasion surprising too. He had the most penetrating judgement of men and their motives, and his advice, sought by many in private as well as in public affairs, was invariably shrewd and solid. Inevitably he had a large circle of friends, and he was an indefatigable and lively correspondent, increasingly so after his transfer to Dublin. His letters were the kind that one keeps—informative, amusing, gossipy, often cryptic and not infrequently pungent in their comments on local and other affairs. It may confidently be said that John Kay Jamieson will always be gratefully remembered by his pupils, his colleagues and his friends as a notable and remarkable figure, whom to have known has indeed been a liberal education.

Professor Jamieson married Elisabeth, daughter of Dr R. P. Goodworth. Her kindly welcome is a happy memory of all former members of her husband's Staff at Leeds. She died there in 1936—her loss may well have affected his decision to move to Dublin—and they leave a daughter and a son, Dr John Jamieson, to whom we extend our sincere sympathy.

J C B
R G I



GEORGE LINIUS STREETER

IN MEMORIAM

GEORGE LINIUS STREETER, A B , A M , M D , D Sc , Sc D (Dublin),
LL D (Michigan), F R S Edmb

Sometime Assistant in Anatomy, Johns Hopkins University
Professor of Anatomy, Wistar Institute, Philadelphia
Professor of Anatomy, University of Michigan
Research Assistant, Carnegie Institution of Washington
Director of the Carnegie Laboratory of Embryology, Baltimore
Member of many Learned Societies
Honorary member of the Anatomical Society of Great Britain and Ireland

The sudden death, at the age of 75, of Dr George Linus Streeter has removed from the contemporary field of human embryologists its greatest figure. Dr Streeter was trained in the great tradition of descriptive embryology and neurology and his earlier work, summarized in Keibel and Mall's Manual, was concerned largely with the development of the human peripheral and central nervous systems. After 1914, however, when he left his position as head of the Anatomy Department at the University of Michigan to go to a full-time research post at the newly founded Carnegie Laboratory of Embryology in Baltimore, his embryological interests became much broader, or, at least, found an opportunity to reveal themselves more fully. Following the tragic death in 1917 of Prof. Mall, Dr Streeter became Director of the Laboratory which had already a great reputation, and which will always be associated with the name of its great founder Dr Mall. Under the directorship of Dr Streeter the laboratory grew from the unachieved brilliance of Mall's concept to its present position as the most important laboratory of embryology in the world.

This fulfilment of Mall's dream was due to Streeter's vision, energy and personality. He saw that such a laboratory must be more than a repository of human embryos. To fulfil the aims of the founder the experimental and comparative methods must be added to the descriptive approach, and the basis of embryological study—sex and reproductive physiology—had to be explored. Finally, in the background of all the work there had to be a broad biological interest in embryos as living organisms with historical impulses (which, for Streeter, were genetical) and structural adaptations necessary for coping with, or exploiting, the environment.

To achieve these ends Streeter gathered round him a brilliant group of investigators, the Lewises, Corner, Wislocki, Heuser, Hartman, Metz, Gey, Burn, Flexner. These, with collaborators from the Johns Hopkins Department of Anatomy, Hines, Schultz, Straus, Gersh, and a host of visiting workers from all over the world, under Dr Streeter's guidance, engaged in problems the full extent of which can be studied in *The Annual Report of the Director of the Department of Embryology of the Carnegie Institution of Washington*, and in the *Contributions to Embryology* of which vols VIII to XXIX appeared under his editorship and which 'with self-effacing devotion, with adherence to the highest standards of technical perfection, and with the capacity

to enlist skilled collaboration on the part of scientific contributors, illustrators, printers and engravers, and the Institution's Office of Publications, made a veritable treasury of embryological progress, famous among the world's archives of science' (to quote from the prefatory note to vol xxx of the *Contributions*, dedicated to him by his colleagues and other workers)

While he was concerned with providing facilities for all these workers, with the editing of their publications, with his duties as administrator of a large laboratory and with expanding the research collection of human embryos, Dr Streeter found time to do a great deal of personal investigation, either alone or in collaboration. His descriptions of the development of the internal ear and the cranial sinuses are classical. His surveys of early human developmental stages and of the Miller ovum constitute the basis for all the beautiful Hertig and Rock material which has revolutionized our knowledge of the early development of man. His work with Dr Heuser on the early stages of the pig and, especially, of the *Rhesus* monkey are fundamental. His last publications, on human developmental horizons, are characterized not only by a meticulous accuracy, but by a breadth of biological vision which is a revelation of the way in which embryology should be studied and presented. To these achievements can be added many other significant studies, such as those on twinning, developmental abnormalities and metamerism of the central nervous system.

To conduct a department of this magnitude, to carry out personal research, to attract workers from all over the world, to welcome and inspire them, required a man with a high sense of duty, a love for his fellows, a spirit of enthusiasm, unselfishness, simplicity and friendliness, a readiness to see another's point of view, and a sense of humour. These were the characteristics of Dr Streeter.

The photograph was taken by Mr F J Pittock in Prof J P Hill's laboratory

J D B

C M W

Anatomical Society of Great Britain
and Ireland

PROCEEDINGS

November 1947

April 1948

February 1948

June 1948

RECORDED BY
W F HARPER

PROCEEDINGS OF THE ANATOMICAL SOCIETY

NOVEMBER 1947

The Annual General Meeting of the Society, for the Session 1947-48, was held on Friday, 28 November 1947, in the Anatomical Department, University College, Gower Street, London, the President (Professor A B APPLETON) in the Chair

Professor J Z YOUNG presented a communication on 'Narrowing of nerve fibres at the nodes of Ranvier'

Measurements of the thickness of the axon and myelin in serial sections through nodes of Ranvier of rabbit's nerves show that the axon becomes reduced, over a length of about 25μ , to as little as a quarter of the diameter it shows along the internode. This constriction is produced by an increase on each side of the node in the thickness of the myelin, which may reach 7.5μ compared with 2.5μ over the rest of the fibre. The axon is thus reduced at this point to a narrow channel, about 5μ in diameter in the case of a large fibre, surrounded by a myelin sheath of 7.5μ thick. The axon also remains narrow in the node proper, the region where it is not surrounded by any myelin, the length of this being about 5μ in the case of the largest fibres.

The great narrowing of the fibre at the node, together with the increase here of the myelin, may be responsible for some of the membrane like properties which have been recorded as observable electrically at the nodes. No visible transverse membrane has been seen crossing the axon itself at the node, but the axoplasm may show slight differences of staining where it becomes narrow. The inturning of the neurilemma to make the cementing disc can also give appearances difficult to distinguish from those of a transverse membrane across the axon.

Professor LE GROS CLARK inquired if the constrictions observed in the preparations might not be artefacts, and Professor YOUNG replied that they did not occur elsewhere on the nerve fibres, and other workers had recorded similar appearances.

Professor MACCONAILL asked if narrowing of nerve fibres had been noted in other mammals, and was told that they had been observed but that the relative measurements of nodal and internodal regions differed in the various orders of Mammalia.

Dr WEDDELL inquired if there was any significant variation in the degree of narrowing of the fibres from one rabbit to another, and Professor YOUNG replied that he had not gone into this matter fully but that in the nerves of frogs he had observed some variation.

Dr J JOSEPH read a paper on 'Quantitative nuclear changes in the peripheral stump of the greater splanchnic nerve of the rabbit'.

The greater splanchnic nerve of the rabbit is composed almost entirely of small myelinated fibres. In normal nerves the number of nuclei in right and left nerves and at two levels in the same nerve is not significantly different. There is, however, a significant difference between individual rabbits. After 21 days' degeneration, the peripheral stump shows an increase in the number of nuclei of the order of only 34%, as compared with 800% in a large myelinated nerve, and with 0% in a non-myelinated nerve. These differences may be explained by the quantity of degenerating myelin and/or the size of the fibres. Previous investigators attempted to relate the proliferation of the cells in the peripheral stump to regeneration, the proliferation being necessary either for a pathway for the growing axon or to produce myelin for the maturation of the fibres. At 21 days it is impossible to determine to which type of cell the increase in number is due.

Professor YOFFEY inquired if the author had made any specific tests for macrophages following degeneration. Dr JOSEPH replied that while it was impossible to distinguish between the different types of cell in a transverse section of the greater splanchnic nerve

at the end of 21 days' degeneration, two types of cell could be recognized after 1 week of degeneration. Comparison of the two might determine to what extent different cells had increased. He added that other investigators have stated that there is no increase in the number of macrophages in relation to degenerating small fibres.

Dr L. CHACKO read a paper on 'The laminar pattern of the lateral geniculate body in Man and the lower primates'.

Reconstruction models of the geniculate body in Man, *Macaca*, *Lemur*, and *Microcebus* have been prepared. They show that in the primates the geniculate body varies from the completely 'inverted' type in which the small-celled laminae are contained within the concavity of the large-celled laminae, to the completely 'everted' type in which the large-celled laminae are contained within the concavity of the small-celled laminae. The laminar pattern in Man has been studied in relation to the projection of the central and peripheral fields of the retina on the geniculate body, and also with special reference to the distribution in the retinal field of colour sensitivity. Finally, the total number of cells in each set of laminae of the human geniculate body has been computed from serial sections, from which it appears that the ratio of crossed to uncrossed fibres in the optic tract is approximately 60 to 40.

Professor LE GROS CLARK, commenting on the functional significance of the laminar pattern, emphasized that a recording of electrical responses from localized areas of the geniculate body during the stimulation of the retina with monochromatic light might help in the elucidation of the problem. Dr CHACKO remarked that a record of the areas of degeneration of the geniculate nucleus after prolonged exposure of the retina to monochromatic light, and a detailed analysis of the nucleus in cases of colour scotoma might also help.

Dr W. H. FEINDEL read a paper on 'The pattern of innervation in the muscle-spindle of the rabbit'.

Using a modification of the intravital methylene-blue technique it has been possible to demonstrate the entire pattern of the intramuscular nerve supply of a lumbrical muscle in the rabbit. In such a preparation it is possible to study the relationship of the various nerve fibres and endings concerned with the innervation of the muscle spindles, for the muscle is small enough to allow complete examination without sectioning.

It has been found that the muscle-spindles are supplied by one large myelinated nerve fibre and by at least four smaller fibres, and the distribution of these in relation to the intrafusal muscle fibres has been defined. The large myelinated fibre, shown by Sherrington (*J. Physiol.* 1894, 17, 211-250) to be afferent in nature and by Lloyd (*J. Neurophysiol.* 1943, 6, 317-326) to subserve the stretch reflex, has been traced to a single fibre directly from one of the muscle-spindles to the main nerve bundle. The terminations within the muscle-spindle of the smaller nerve fibres show considerable variation in morphology and in many cases are supplied by at least two axons.

Dr WEDDELL congratulated the author on his development of the new intravenous methylene-blue method for studying the nervous system.

Dr BARKER asked if the diameters of the large afferent nerve fibres both at the spindle and in the main nerve to the muscle had been measured. Dr FEINDEL replied that while he had not yet made careful measurements there seemed to be no great discrepancy in diameter as one traced them from the spindle to the main muscle nerve. They remained throughout about twice the diameter of the next largest fibres and were, therefore, the only possible fibres which could act as the rapidly conducting afferent arc of the stretch reflex. He also pointed out that the arrangement of a large afferent fibre and motor fibre running together without branching from the end-organ to the main muscle nerve was found as well in relation to the tendon-organ, and that either or both of the organized endings might be concerned in the stretch reflex.

Dr C H A WEDELES (introduced by Professor J Z YOUNG) read a paper on 'The effect of increasing the functional load of a muscle on the composition of its motor nerve'

This experiment is part of an investigation designed to ascertain the influence of peripheral connexions on the structure of motor fibres. It is now known that the peripheral, as well as the central connexion, is an essential contributor to the restoration of injured nerves. Normalization of fibre number and diameter proceeds imperfectly with severed or non functioning distal connexions.

The medial belly of the gastrocnemius muscle of the rabbit on one side was made to perform extra work by denervating the lateral belly, plantaris and soleus, the other side serving as control. After 100 days the overloaded normal nerve showed an increase both of the total number of its constituent fibres and in its total fibre area. There is a shift of fibres into larger size groups, and previously 'non-medullated' fibres acquire myelin sheaths.

The effect of overloading a nerve during regeneration was also studied by crushing the nerve to the medial head of gastrocnemius on both sides and denervating the other muscles as before on one side only. After 100 days of regeneration the overloaded nerves showed a considerably greater number of fibres, but these were not larger than normal.

Professor WEST inquired as to the source of the additional nerve fibres and was informed that the author was quite ignorant as to their origin or function. Dr WEDELES suggested, however, that either the so called 'unmyelinated' fibres had acquired a thicker myelin sheath and so become visible by the Weigert-Pal technique, or that there had occurred a branching of existing fibres in response to muscle hyperplasia. There are no facts to prove either assumption and further work must be done to elucidate the problem.

Professor YOUNG remarked that in any case the increase in the number of new nerve fibres was not large.

Dr B FRANKENHAEUSER read a paper entitled 'A method of determining the cutaneous distribution of a sensory nerve by means of action potential recording'.

The cutaneous distribution of the saphenus minor (sural) nerve in the rabbit has been determined by recording the action potentials produced in the nerve following stimulation of the hairs. The hairs were stimulated with a no. 3 nylon suture mounted in a metal holder. The action potentials were led off from the exposed nerve by two silver-silver chloride electrodes feeding into separate amplifiers. Stimuli outside the area of distribution of the nerve gave rise to no action potentials, but when stimuli were applied to the autonomous zone several spikes were recorded. Stimuli applied to the intermediate zone produced a few spikes only. It appears that when the nerve has suffered no damage every spike visible in the record from one electrode is also visible in the record from the other. The cutaneous distribution of the saphenus minor nerve so outlined is in general agreement with that previously found by Weddell, Gutmann & Gutmann (1941).

It is submitted that this method of outlining sensory areas in the skin, in which the physiological integrity of the nerve and its cutaneous end-organs is preserved, is more valid than the older destructive procedures, and therefore has a place in the anatomical armamentarium.

Dr WEDDELL and Professor J Z YOUNG commented on the clinical importance of this new method for demarcating sensory areas in the skin.

Professor M A MACCONAILL read a paper on 'The myelothecal apparatus of the axis cylinder'. He described a method of staining the C.N.S. with lead as follows.

The myelothecal apparatus is stained with complete regularity by the method described below. It consists of a thin membrane, seemingly continuous, forming an immediate sheath to the medullated part of the axis cylinder, i.e. it is internal to the myelin. This is the primary part. The secondary part consists of a well marked spongework, extending across the myelin space, and forming a reticulum on the surface of the myelin sheath proper. It appears to be elastic. This part is specially developed to form the infundibula at the incisures of Schmidt-Lantermann. The primary part is present within and without the brain and

cord, but the secondary part is found in peripheral nerves only. The development of the primary part appears to proceed *pari passu* with medullation.

Paraffin sections are brought through the alcohols to a saturated solution of picric acid (2 min). They are then immersed in acid fuchsin (1 %, 10 min) and then in lead haematoxylin (made by mixing equal parts of aqueous solutions of haematoxylin (2 %) and lead nitrate (5 %), tap water may be used, the mixture is filtered and allowed to ripen for 1 or 2 days), (4 min). Lastly, they are placed in saturated ammonium molybdate solution for 1 min. The sections are, of course, washed well (tap water) between each of the foregoing stages. The times given are for 6μ sections, they should be halved for 12μ sections, except for the ammonium molybdate bath. Results: nerve cells and dendrites, dark brown, nucleoli of nerve cells, red, axis cylinders, dark blue to black, myelothecal apparatus, bright red, collagen, dull pink, red blood corpuscles, rose-red.

Dr R. G. HARRISON remarked that he had hitherto agreed with Nageotte that networks of the character demonstrated were artefacts, but now thought otherwise as a result of the series of differences between the various types of nerve fibres shown by Professor MACCONAILL.

Professor H. BARCROFT and Dr C. T. C. HAMILTON (introduced by Professor J. Z. YOUNG) read a paper entitled 'Results of sympathectomy in the upper limb'.

(1) Examination of seventeen upper limbs 1-6 months after sympathectomy (preganglionic section) showed (a) Marked clinical improvement. No recurrence of the attacks in fourteen out of sixteen hands operated on for Raynaud's disease. In two hands the attacks were less frequent and less severe. (b) That vasometer and sudomotor reflexes were absent. (c) That therefore preganglionic section equals complete sympathectomy of the limb. Th. 1, which is not cut, does not convey sympathetic fibres to the hand.

(2) Examination of a further thirty-six upper limbs 1-6 years after preganglionic section showed (a) Marked clinical improvement. No recurrence of vasospastic attacks in eighteen out of thirty-six limbs operated on for Raynaud's disease. In fourteen hands the attacks were much less crippling. In only four had no benefit been obtained. (b) That vasomotor and sudomotor reflexes were absent in some limbs, but present in many. (c) That nervous connexion between brain and the vessels and sweat glands of the hands is often re-established. (d) That the persistence of the excellent clinical results in spite of the re-establishment of a vasomotor path probably signifies that the new path is functionally inferior to the original one. (e) That partial regeneration is the most likely explanation of the new nerve path.

Mr K. C. RICHARDSON read a paper on 'The problem of contractile tissues in the mammary gland'.

Four components of the mammary stroma may be involved in varying degrees in the mechanical transfer of milk from the alveoli to the ducts and cisterns, but recent physiological investigations suggest that the myoepithelial cells, associated with the alveolar epithelium, are the main contractile component, and that smooth muscle bundles, elastic fibres and blood vessels are of minor importance. The position, number and morphology of the myoepithelial cells in the goat mamma have been examined in preparation for further experiments on the structural changes in the udder associated with the phenomenon of 'let-down'.

Dr GLEES inquired whether there was not some possibility of confusion between reticular fibres and myoepithelial cells. Some of the silver preparations shown reminded him of stained argentophile fibres and he wondered how one was able to discriminate between these and contractile elements. Mr RICHARDSON replied that the structures outlined by silver impregnation in the mammary gland were undoubtedly cells, similar in size, distribution and location to the myoepithelium revealed by routine staining. Moreover, the silver technique could be controlled by adjusting the pH so as to stain myoepithelium, smooth muscle and connective tissue fibres separately without any possible confusion.

Dr D H L EVANS read a paper on 'Some problems in enteric innervation'

The enteric plexuses of the cat, rabbit and frog stained with Bielschowsky-Gros, Cajal and methylene-blue methods show that the interstitial cells of Cajal occur not only in relation to the enteric plexuses but also throughout the circular muscle coat. They form the web like structure of the plexuses in which the nerve fibres run, and thus resemble Schwann cells. Beyond the plexuses methylene-blue preparations show nerve fibres running in the processes of interstitial cells in their course towards the muscle elements. Cajal preparations of the circular muscle show columns of nerves running parallel to the muscle fibres and distributing branches which run close to the muscle cells. No undoubted endings were found, and it seems possible that a functional synapse is established over an extensive length of nerve fibre as it runs adjacent to several muscle cells. No peripheral network was seen, such as would be expected on the theory that post-ganglionic fibres anastomose to form a syncytium at their terminations.

Dr GLEES commented on Stoehr's conception of the terminal reticulum. They were not artefacts as had been maintained by Noidetz but represented terminal nervous arborizations. The Cajal preparations demonstrated by Dr Evans did not show a complete staining of all nerve fibres. Cajal's technique was unsuitable for the peripheral nervous system. The only hope of getting some agreement in this difficult field of research would be for the two opposing schools to meet and discuss their conceptions.

Professor SOSA said that in silver preparations, either with Cajal, Bielschowsky, Gros or Golgi methods, or in Ehrlich's methylene-blue preparations, he had never found the so called 'terminal reticulum' as a real nerve structure. When it did appear under some technical conditions, it could be interpreted as the result of a partial impregnation of pre collagen fibrils. On the other hand, he had always seen the terminal innervation of smooth muscle fibres, in the intestine and other organs, made only by contact of terminal knobs or masses on the surface of the muscle cells as stated by Cajal.

Dr BRANDT stated that in a recent monograph Hillarp concluded that the 'terminal reticulum' of a nerve fibre does not exist. In Auerbach's plexus in the gut of *Myxine* after the application of silver nitrate (Schultz-Gros method) ganglionic cells and nerve endings similar to those Dr Evans had shown, can be demonstrated. But there is no peripheral network.

Dr E C AMOROSO in a paper entitled 'The vascular and endometrial relations of the placenta of the sow' discussed the manner of union of the trophoblast and the mucosa.

Dr BRANDT remarked that in a recent number of the *Revue Suisse de Zoologie* Tondury had described the placenta of the sow in detail, noting specially the close relationship of the blood capillaries to the epithelium.

Professor NICOL congratulated the author on the excellence of his histological preparations and photomicrographs.

Dr A LASVITZKI (introduced by Professor S ZUCKERMAN) read a paper on 'Investigations on the conversion of lymph-nodes into haemolymph nodes'.

While the occurrence of haemolymph nodes in the normal rat is confined to the renal and splenic regions, it has been found that, under experimental conditions, lymph-nodes in various other regions of the body can be converted gradually into haemolymph nodes of more or less pronounced character. Changes of this nature were brought about, first, by the influence of a prolonged treatment with the carcinogenic hydrocarbon 1,2,5,6-dibenzanthracene, administered subcutaneously in the form of an aqueous colloidal solution. The haemolymph nodes thus produced corresponded in their gross anatomical appearance and histological structure to those already present in untreated rats. Similar, although less striking, results were obtained if rats were treated similarly with two other carcinogenic hydrocarbons, 3,4-benzpyrene and methylcholanthrene. On the other hand, the two non-carcinogenic hydrocarbons anthracene and phenanthrene, administered in a comparable manner, failed to produce any appreciable haemolymphatic changes.

It has been observed, further, that abnormally situated haemolymph-nodes, with similar morphological properties, also occurred in rats bearing transmissible tumours implanted under the skin (Jensen sarcoma). This action was supposed to be due to a substance released by the growing tumour, and subsequent experiments, undertaken from this point of view, showed that significant haemolymphatic changes, although less frequent and pronounced, could be obtained after subcutaneous administration of the total lipoids extracted from dried tumour tissue. The total lipoids contained in samples of human cancerous tissue had similar effects.

Dr J WHILLIS and Dr G D CHANNELL presented a paper on 'The action of the lumbrical and interosseous muscles in some of the movements of the digits'.

Though for the past one hundred years the accepted view has been that the lumbricals produce extension of the interphalangeal joints by a direct pull on the extensor expansion, this is not really the case.

There are two natural movements in which extension of the interphalangeal joints occurs, viz (1) opening the hand when extension occurs also at the metacarpo-phalangeal joints, and (2) the movement used in threading a needle where extension of the interphalangeal joints is accompanied by flexion of the metacarpo-phalangeal joints.

In opening the hand the movement is produced by active contraction of the extensor digitorum communis accompanied by reciprocal relaxation of the flexor muscles.

In the 'needle-threading' movement the extensor communis and the flexor muscles both maintain a constant tone. Flexion of the metacarpo-phalangeal joint by the lumbrical and interosseous muscles produces automatic slackening in the flexor tendons and tightening of the extensor communis which is expended in extending the interphalangeal joints, the role of the long tendons being that of inextensible passive ligaments.

The lumbricals also produce radial deviation and rotation of the digits when they are opposed to the thumb.

Their role in relaxing the flexor tendons in unnatural movements with the first phalanx fixed was discussed.

The results have been checked by the production of acute paralysis by injection of the radial and ulnar nerves and by direct stimulation of the lumbricals.

Professor MACCONAILL expressed his satisfaction at the facts established, they were confirmatory of conclusions drawn from the theoretical mechanics of the joints.

Dr J MCKENZIE read a paper on 'The parotid gland in relation to the facial nerve'.

McWhorter (*Anat Rec* 1917, 12, 149-154), describing the parotid gland as a bilobed structure, was the first to state that the larger lobe, lying superficial to the facial nerve, and the smaller, deep to the nerve, were joined by an 'isthmus' consisting of gland parenchyma or ducts or both, which passed between the two main divisions of the nerve, the temporo-facial and cervico-facial. Recently Hamilton Bailey (*Brit med J* 1947, 1, 404-407), confirming these views, stated 'There can be no doubt that surgical enterprise in the parotid region has been stultified by the instruction that surgeons have received and are receiving from anatomists'.

The present series of dissections which, it is hoped, will refute these charges, shows that further communications occur between the two portions of the gland in addition to those previously described. Small ducts have been observed passing between the smaller branches of the facial nerve from lobules of the gland lying deep to the nerve, lobules which have been accepted as part of the deep 'lobe' of the gland by McWhorter, Roubiere & Cordier (1934), McCormack, Cauldwell & Anson (1945) and others.

The PRESIDENT said that he agreed with the conclusions drawn from the dissections and raised the question as to the relation of the parotid gland to the facial nerve in early foetal life. Dr MCKENZIE was unable to give any information on this point, as he had not studied this aspect of the problem.

Dr R J HARRISON read a paper on 'The vascular supply to the human testis'

The blood supply of the testis as demonstrated by radiography and micro-arteriography was described. The common statement that the testicular artery passes into the substance of the testis at the mediastinum is found not to be true. The commonest arrangement is for the artery, on reaching the posterior border of the testis, to divide into two branches, both of which pass into the tunica albuginea to ramify in the tunica vasculosa over the lateral and medial surfaces respectively. The terminal branches are given off from these superficial branches at various points over the free surface of the testis and pass in towards the mediastinum, commonly bending back again to pass centrifugally before reaching it. The veins draining the testis are arranged as small, tortuous, superficial veins, deep veins, and large short-circuiting anastomotic channels.

Reference was made to the vascular pattern in the testis of other animals, particularly the wallaby, *Macropus rufogrisea fruticus*, in which a sort of 'rete mirabile' is formed in the spermatic cord by the testicular artery.

Professor CAVE inquired whether any indication of the distribution of the artery to the gubernaculum in the testis had been observed. Dr HARRISON replied that this had not been seen, but that the next obvious problem was the elucidation of the distribution of other arteries, e.g. the artery of the vas.

Dr R BARER read a paper on 'Phase contrast microscopy in Anatomy'

Some applications of phase contrast microscopy to anatomical problems are described. The method is invaluable for the examination of living material both as isolated cells or in the living intact organism. Transparency, rather than actual thickness of the specimen, is the limiting factor in its use. Contrary to what is often stated, the method may be extremely useful as an accessory to routine examination of fixed and stained sections. Elements which may have been poorly stained can often be rendered clearly visible. A detailed investigation of the effects of fixatives and routine histological procedures on living cells is now in progress.

Professor MACDONAILL remarked that the microphotographs of nerve cells and of the cerebellar cortex shown were remarkably like his own preparations obtained by the lead haematoxylin technique described earlier in the meeting.

Dr W F HARPER read a paper entitled 'Further observations on the blood vessels of the nasal mucous membrane in mammals'

In a previous communication the author has reported the presence of arterio-venous anastomoses in the human nasal mucosa. Observations are now presented on the location, relative number and structure of these anastomotic cross connexions between the arterioles and the venous blood spaces in the mucosa of the human inferior turbinate. Additional data concerning the structure of the walls of the venous blood spaces, the intra-osseous veins of the inferior turbinate and the so called 'Drosselvenen' of Korner (*Z mikr Anat Forsch* 1936, 41, 131) are given and discussed with reference to the erectile capacity of the mucosa.

The blood vascular patterns of the turbinate mucosa in the cat, rabbit and certain ungulates are described and compared with that of Man.

An interesting type of arterio-venous communication occurs in the turbinate mucosa of *Neotragus* and *Mouflon* (Ungulata). In these forms the lumina of arterioles open *directly*, through a valvular mechanism in the wall of the arteriole, into large venous blood spaces without the intervention of a specialized connecting channel.

The PRESIDENT commented on the large size of the blood spaces in the human nasal mucosa, and Professor LE GROS CLARK suggested that there might be some relationship between the blood vascular channels of the mucosa and the olfactory nerves.

Dr G H BOURNE read a paper on 'Alkaline phosphatase and vitamin C deficiency in regeneration of skull bones'

Holes, 1 mm in diameter, were bored in the parietal bones of the skulls of guinea pigs and

the holes permitted to heal for periods varying from 24 hr to 2 weeks. Undecalcified sections were cut through the skull passing through the healing hole. Alkaline phosphatase (Gomori's method) preparations were made of these sections. Some of the animals were daily injected intraperitoneally with trypan blue for a week preceding operation and until autopsy. These experiments were also repeated with guinea-pigs which had been on a scorbutic diet for a week prior to operation.

The results showed that there was an accumulation of cells resembling polymorph leucocytes, which gave an intense positive phosphatase reaction, at the site of injury. Osteoblast-like cells and cells which appeared to be forming capillary vessels were also present. They were phosphatase positive. Macrophages were also present in large numbers in the injured area, they gave no phosphatase reaction.

At 3 days there was a considerable decrease in the number of polymorph-like cells in the injured area, but numerous phosphatase-positive fibres and some positive capillaries were present. At 1 week massive concentrations of phosphatase in the injured area heralded the onset of the trabeculae.

Guinea-pigs on a scorbutic diet showed a reduction not only in the production of fibres but in the number of polymorph-like cells and macrophages present in the injured area. The ability of those polymorph-like cells which were present in the injured area to give the phosphatase reaction was unaffected by the scorbutic condition.

Dr WYBURN stated that bone continued to form even after treatment with cyanide which is presumed to inactivate phosphatase. Dr BOURNE replied that he was not directly concerned with this problem and he did not deny or assent that phosphatase was concerned in bone formation.

Dr A. SLESSOR and Dr G. M. WYBURN read a paper on 'Bone formation'.

The occurrence of bone formation in the host tissues (guinea-pig), following the presence of bone homografts in the subcutaneous tissues for 5-6 days, was previously reported (Wyburn & Bacsich). Bone homografts consisting of the femoral epiphysis (bone and cartilage) were implanted and maintained in the subcutaneous tissues of guinea-pigs for 3 weeks. There was no evidence of bone formation by the tissues of the host.

Bone homografts (pieces of shaft of femur) were implanted into the flexor muscles of the hind limbs of rabbits. After 4 weeks there was no evidence of bone formation by the host tissues.

Blum (1944) asserts that he obtained formation of bone in rabbit muscle after the intramuscular injection of phosphatase. His experiments were repeated and, in addition, pellets of phosphatase were implanted intramuscularly and left for 4-5 weeks. There was no evidence of bone formation.

Dr BOURNE, in support of the evidence presented, stated that he had introduced extracted rabbit phosphatase into holes drilled in rabbit femora, in others he had covered similar holes with filter-paper containing rabbit phosphatase, in others he had implanted phosphatase in powder form and on filter-paper subcutaneously, he had also implanted pieces of alcohol-fixed urinary bladder and gall bladder into and over holes in rabbit femora and in no case was there any apparent action on bone formation.

Dr P. BACSICH and Professor W. J. HAMILTON read a paper on 'Supravital staining experiments on the tubal ova of the rabbit'.

1/10,000 aqueous solution of toluidin-blue was applied as supravital stain to living tubal ova of the rabbit. Sections of tubal eggs were also stained with toluidin-blue.

The 'albuminous' coat gradually deposited on the rabbit's ovum as it passes down the tube gave an intense metachromatic staining in all of the experiments, thus indicating that it is mainly composed of muco-proteins. Staining results also suggest in the tubal eggs the presence of a slow enzymatic hydrolysis of this muco-protein coat immediately outside the zona pellucida, and a more rapid hydrolysis contemporary with, and probably mainly responsible for, the rapid expansion of the blastocyst when it reaches the uterine cavity.

These supravital staining experiments, probably the first ever carried out on living mammalian ova, also revealed the existence in the tubes of large muco-protein spherules, structures closely resembling those found by Hartman (1916 and 1919) in the opossum

The significance of these findings was discussed

FEBRUARY 1948

An ordinary meeting of the Society, for the Session 1947-48, was held on Friday, 20 February 1948 in the Department of Anatomy, The Medical School, Hospitals Centre, Birmingham, the President (Professor A B APPLETON) in the Chair

Dr T L KRON and Professor S ZUCKERMAN read a paper entitled 'Stimulation of the seminiferous epithelium of immature monkeys with androgens'

Androgens stimulate and oestrogens depress the germinal tissues of immature female monkeys. In view of this observation it was of interest to reinvestigate the effects of the same hormones on the immature monkey testis. It is confirmed that oestrogens depress the size and activity of the seminiferous tubules. Androgens, on the other hand, have a stimulating effect, although, as previously reported, sperm formation has not been observed in our experiments. Our new observations can be related to comparable findings on rats, and on hypophysectomized mature monkeys.

Professor ZUCKERMAN pointed out that the same material on which he and Dr KRON were reporting had been investigated by him alone earlier, in order to discover whether androgen stimulated spermatogenesis in immature monkeys. The results of the earlier investigations were negative, but no attention had been paid to detailed changes in the first stages of germinal development.

Professor BOYD congratulated the authors on their findings and referred to the work of Tondury on the effect of androgens on the speed of cleavage in growing eggs.

Dr A P D THOMSON and Professor S ZUCKERMAN read a paper on 'The vascular and nervous connexions of the retina concerned in the response of the gonads to light'

Previous experiments designed to determine the optic pathways along which light stimulation of the retina affects the gonadotrophic potency of the ferret pituitary have led to equivocal results. The problem is being reinvestigated in order to discover first, whether the initial stimulus is a nervous one, and second, whether it can be elicited with the pituitary transplanted away from the sella turcica. Four groups of animals have been used: (a) animals in which only the optic nerve fibres have been divided, and in which the central artery of the retina, and the long and short ciliary vessels and nerves are left intact, (b) animals in which the optic fibres are not disturbed, but all these other structures are crushed, (c) animals in which both sets of connexions are divided (as in all previous corresponding experiments in which the 'optic nerves' have been divided), and (d) controls. The results of this experiment show that the gonadotrophic response is dependent on the integrity of the optic nerve fibres themselves.

Professor BOYD inquired as to the presence or absence of myelinated and non myelinated fibres in the optic nerve. Dr THOMSON replied that no histological examination had been made.

Dr BOURNE asked if the authors had any information regarding the effect of light on the reproductive cycle of monkeys that do not have a long seasonal period of anoestrus. Professor ZUCKERMAN replied that there was no information, to the best of his knowledge, about the influence of light on the reproductive cycle in monkeys, but that it had been shown by Broman that in the rat optic enucleation causes a marked delay in the various phenomena characteristic of normal growth and sexual maturity.

Dr GLEES inquired concerning the difference between 'optic nerve fibre cut' and 'optic nerve cut'. Dr THOMSON answered that in the former the sheath of the optic nerve and all the contents of the sheath are damaged as little as possible, and only the fibres of the optic nerve are severed. By this means only the central neural connexions between the eye and the

brain are involved and the circulatory connexions are preserved. In the case of optic nerve cut the sheath and its contents along with the optic nerve fibres are cut and no blood or nervous connexions between the retina and the brain are left.

Professor YOFFEY inquired about the possibility of degenerative changes following section of the optic nerve. Dr THOMSON replied that while no histological examination had been made he expected to find that a degeneration of the ganglion cell layer of the retina had occurred, and he cited cases in man in which this had been noted following certain haemorrhages, e.g. post-partum, melaena, etc.

Professor GOLDBY remarked that he did not consider that Jefferson's negative findings in the ferret, so far as hypothalamic connexions of the optic nerve were concerned, were entirely convincing. He had himself observed aberrant optic fibres in the sheep which, in some cases, run towards the pre- and supra-optic regions. These fibres were not traced to their termination, and it may be that they return to the optic tract although they have not been seen to do so. In his opinion the question of hypothalamic optic connexions was still an open one.

The PRESIDENT congratulated the authors on their technical skill during these delicate operations on the optic nerve.

Dr I. C. MICHAELSON (introduced by Professor W. J. HAMILTON) read a paper on 'Vascular morphogenesis in the retina of the cat.'

A study of injected retinae of foetal and adult cats shows the following:

(1) Vessel growth in the retina of the cat is by a process of budding from pre-existing vessels. No evidence could be found of vascular differentiation from local cells.

(2) The formation of retinal capillaries is pre-eminently a function of the retinal veins. Only the arteriae afferentes appear to originate from arteries.

(3) If vein and artery are close to each other, growth takes place initially from the side of the vein remote from the neighbouring artery.

(4) The spread of capillary growth towards an artery extends for only a certain distance, leaving a well-marked capillary-free space around the arteries similar to that present in other mammalian retinae such as those of Man, dog, rat and pig.

(5) These anatomical facts are clearly associated with each other. Considered as a group they suggest the presence of a factor or factors which affect the growth of retinal blood vessels.

(6) The present ontogenetic study shows that the bulk of the capillary system in the retina of the cat can be considered as part of the venous system. The arterial system is shown to be supra-capillary in origin.

Dr BOURNE asked if the growth of the regional capillaries might be due to the action of inhibitory substances released. Dr MICHAELSON replied that this was a possibility. Dr BRANDT commented that the author's injections showed only those capillaries whose calibre allowed the injection mass to enter. The early solid endothelial outgrowths from the arteries cannot be demonstrated in this way. He suggested a further histological examination.

Dr WYBURN remarked that environmental differences might affect the new growth of the capillary network.

Dr B. H. DAWSON read a paper on 'The blood supply of the optic chiasma.'

Since neurologists at the present time favour chiasmal pressure ischaemia as the probable cause of visual field defects in patients with tumours in the chiasmal region, it is necessary to establish the exact blood supply of the optic chiasma.

The details given of the chiasmal 'Epineural Vessels' were based on dissections of 230 fresh P.M. brains, and in order to avoid tearing fragile vessels the greater part of the sphenoid was removed with the brain *en bloc*.

The intimacy of association between the blood vessels of the chiasma, and those of the hypophysis and hypothalamus was described and illustrated and another arterial anastomotic system with the circulus arteriosus was demonstrated.

The foramina through which the vessels entered the substance of the chiasma were clearly shown, as was also the supraclinoid hypophyseal system and its distribution both along the infundibulum and over the inferior surface of the optic chiasma

Seen clinging to the anterior border of the chiasma was the prechiasmal arcade joined by vessels from the ophthalmic, internal carotid, and anterior cerebral arteries

The many small vessels from the proximal part of the anterior cerebral artery which provides a rich supply to the supraoptic nuclear region were shown and the high vascularity of this region noted

Professor ZUCKERMAN asked for further information about the intimacy of association between the vessels of the chiasma and those of the hypophysis and hypothalamus

Dr DAWSON replied that he had found a very close connexion between the blood vessels of the chiasma, hypophysis and hypothalamus and mentioned how very difficult it was to keep the injection material well localized to any one of these areas. Even with the profuse application of Cushing's haemostatic clips both before and during injection of the coloured gelatine it had been found difficult to fill only the vessels of one of those three systems

Professor HAMILTON remarked that the pressure required to obliterate the complex system of vessels shown would need to be remarkably high, and wondered against which unyielding structure these chiasmal vessels might be compressed. Would not the fibres of the chiasma be damaged before their blood vessels were obliterated?

Dr DAWSON said that the whole question of pressure ischaemia and nerve conduction interference was very unsettled and that he was attempting to elucidate these problems

Dr MICHAELSON favoured the vascular compression mechanism in view of the rapidity of recovery of visual field defects following decompression

Professor YOUNG remarked that the vascular pattern on the chiasma was very like that of peripheral nerve, and inquired whether longitudinal anastomoses had been observed

Dr DAWSON replied that he had been impressed with the similarity in pattern between the vascularization of the chiasma and the peripheral nerves. He could demonstrate the epineural longitudinal vessels in his photographs

Dr W. C. OSMAN HILL read a paper on the 'Blood supply of the human caecum'

Standard descriptions are based upon figures by Jonnesco (in Poirier's *Anatomie humaine*) and subsume a symmetrical arrangement of anterior and posterior caecal arteries. This is not so in lower Primates, with one exception (*Ateles*), and hardly to be expected in Man in view of difference in size of the two vessels. Preparations were shown to indicate that the anterior artery has a relatively limited distribution on the ventral aspect of the basal part of the caecum and on an area opposite the ileal entrance. The rest of the caecum, including the ventral aspect of its fundus, is supplied by branches from the posterior vessel, one proceeding ventral to the root of the appendix. The appendicular artery is independent of both caecal trunks in Hominoidea. A specimen of the rare anomaly of substitution of the dextral for the sinistral supply to the appendix with associated peritoneal anomalies was demonstrated.

Dr BRANDT inquired if any study had been made on the vascularity of the developing human caecum and Dr HILL replied in the negative.

Professor WEST referred to the position of the meso-appendix and asked about the factors concerned in its final disposition on either the ventral or dorsal side of the terminal portion of the ileum, and whether this was in any way associated with the rotation of the gut. Dr HILL replied that usually the meso-appendix was sinistral, but that dextral substitution occurred occasionally in man and orang and was normal in *Lagothrix*, and about equally common with sinistral arrangement in *Aotes*. The factors deciding this are unknown.

Dr GILES inquired whether the vascular arrangements were different in the retrocaecal appendix and was informed that topographical relations had no effect in determining the arterial supply in Man.

Professor KIRK remarked that in removal of the appendix, bleeding from its base is sometimes observed even after ligation of the appendicular artery. He considered that this was from a branch of the anterior caecal artery. In view of the fact that the appendix developmentally is part of the caecal wall this is not surprising.

The PRESIDENT commented on the bloodless fold of Trevès, and Dr HILL, in reply, stated that this fold was much more extensive in the lower primates and was primitively anangious, but that occasionally both in monkeys and Man a recurrent vessel entered it from its dorsal edge derived from the morphologically sinistral side

Dr E W WALLS read a paper on 'An investigation into the regenerative capacity of mammalian heart muscle'

It has been recently demonstrated that striped muscle in rabbits and rats possesses considerable power of regeneration after injury (Le Gros Clark, 1946). Moreover, King (1940) described a case of fatal cardiac injury in Man in which he claimed to have found clear histological evidence of regeneration of myocardial fibres. Accordingly, it was decided to investigate the response of rabbit's cardiac muscle to injury. Lesions were made in the lower third of the ventricular myocardium by the application of the head of a nail heated to a dull red heat, and the animals were allowed to survive for periods ranging from 3 days to 1 month. The injured areas were then studied for evidence of regeneration of cardiac muscle fibres. No such evidence has been obtained. It is thought that the type of lesion used is possibly too severe, producing too great a reaction in the adjacent tissue. Burning was selected as the method as it had previously been used successfully (Thomas & Harrison, 1944) for functional recovery tests in rats, and because it is difficult by crushing to produce comparable lesions in rapidly beating hearts.

Dr E FRANCIS asked whether Dr Walls had seen anything in his preparations that would substantiate the claim of Retzer that ordinary heart muscle developed from Purkinje fibres. The reply was in the negative.

Dr A LASNITZKI remarked that embryonic heart muscle shows in tissue culture pronounced regenerative growth. It would be of interest to know whether, and to what extent, tissue culture experiments have been carried out with heart muscle from adult animals, since the results might have an important bearing on the question of the actual potency for regeneration still existent in myocardial fibres.

Dr FIELD asked whether Dr WALLS believed that intercalated discs did in fact exist in fresh well-fixed, celloidin cut sections of heart. The reply was in the affirmative.

Dr H A MEYLING (introduced by Professor R D LOCKHART) read a paper on 'The structure of autonomic nervous tissue in the right atrium of the heart in some mammals'.

By using the Bielschowsky-Gros silver impregnation method, a dense network of 'autonomic interstitial cells of Cajal' can be observed in the loose connective tissues of epicardium and endocardium. In the endocardium they are closely connected to the Purkinje cells (which, in collaboration with Ter Borg (1939), were found forming a dense network beneath the endothelial lining covering the musculi pectinati, and which are neither connected with the S A node nor the A V node but pass on all sides into the ordinary muscle fibres of the right atrium) and also to the smooth muscle cells which in silver preparations can be seen scattered in the loose connective tissues of the endocardium.

Again, in both the S A and A V nodes, a net of 'interstitial cells', especially well marked in the horse, is found as the most peripheral extension of the autonomic nervous system. They form a lace-work around the small nodal fibres, their branches running alongside these fibres, and in some of them Nissl-substance is found by using the selective stain of Einarson. The difficulty of showing Nissl-substance in the 'interstitial cells' may be due to the functional state of the cell at the moment of fixation, for Einarson has pointed out that during activity the ganglion cell loses its Nissl-substance. Changes in the staining of the nucleus of the 'interstitial cells' (with cap formation on one pole) were found comparable with those seen by Einarson in the ganglion cell, but further experimental research is needed for the interstitial cells, similar to that by Levi, Einarson and others for the ganglion cells.

This network of 'interstitial cells' ought to be identified with the 'sympathetic ground-plexus' of Boeke, and the 'pre-terminal reticulum' of Reiser-Stohr. Lecuwe (1937) has pointed out that these 'interstitial cells' are small anastomosing ganglion cells, containing Nissl-substance. In the carotid sinus and the wall of the aorta, I found (1938) that these 'autonomic interstitial cells' contain Nissl-substance, and are connected with the end

ramifications of the post-ganglionic fibres in an apparently synaptic manner, because their neuro fibrillar network remains intact after experimental degeneration of the post-ganglionic fibres

Accordingly, the sensory nerve endings as described by Smirnow, Dogiel and Woollard in endocardium and epicardium, and by Nonidez and Pannier in the sinuatrial node, are probably the end ramifications of the post-ganglionic fibres forming synapses on the network of the 'autonomic interstitial cells', and the latter are identical with the star-shaped cells and the branching cells described respectively by Dogiel and Woollard in their methylene-blue preparations. Nonidez and Pannier, the former denying the nervous character of the peripheral nervous network, could not in fact demonstrate the 'interstitial cells' as a system, by the silver method they employed.

This conception of the structure of the peripheral autonomic nervous system was obtained in the carotid sinus (1938) by combining the results of silver impregnation and Nissl staining with the vital methylene-blue staining. Now the right atrium has been treated similarly, using vital methylene-blue staining in the modifications given by Schabadasch.

Dr FIELD remarked that the author's description of the large amount of nervous tissue in the heart was of great interest for any theory of cardiac action. Much nervous tissue accompanies the Bundle of His in many animals, and it was of interest to recall the statement of Cohn and Trendelenburg in 1910 that experimental proof of muscular as against nervous conduction in the Bundle had never been achieved up to that time, nor indeed has it since.

Professor BOYD suggested that the cells demonstrated by Dr MEYLING as nerve cells were in reality connective tissue cells.

Dr BRANDT remarked that silver nitrate is reduced in the tissues by very different substances, and we have no methods to hand to prove the metabolism of the tissue responsible for the reduction of silver. Boeke found intrasarcoplasmic endings in the muscle fibres of the heart which differ from the motor endings the author had shown.

Dr MEYLING agreed that sections impregnated with silver need careful interpretation, but with experience it is not difficult to differentiate between connective tissue and nervous structures. In the modification of the silver technique by Gros, by changing the amount of ammonia, the impregnation of connective tissue cells can be entirely suppressed. He had seen autonomic interstitial cells in epi- and endocardium only in places where end branches of afferent or post ganglionic branches were present, and in the preparations of the A V and S A nodes, they appeared only in nodal tissue and not in adjoining musculature. If they were connective tissue cells, these cells would be impregnated everywhere in the connective tissue and in the heart musculature. Moreover, the nervous nature of the autonomic interstitial cells is not merely based on the fact that they are impregnated with silver but on the typical nervous structure they show in methylene-blue preparations.

Dr FRANCIS asked whether interstitial cells were found also in the ventricles, and if so were they similar to the interstitial cells of the enteric plexus. Dr MEYLING replied that they occur everywhere in the heart and that they react to the technique employed in a similar way to those of the enteric plexus.

Dr W. A. FELL read a paper entitled 'The effect of sympathectomy on the size of Haversian canals in the cat.'

In order to induce an increased flow of blood through the bones of the lower limb the lumbar portion of the sympathetic trunk of one side was removed in two cats. Examination of sections of the control and treated tibiae 1 and 10 weeks respectively after operation did not show any gross decalcification on the treated side. The size of the Haversian canals in the compacta of the shaft was then estimated by projection and drawing of their outlines at a fixed magnification. The area of these canal outlines was then estimated by Schmalhausen's method i.e. the outlines were transferred to Bristol board cut out of the board and weighed. By this means a series of estimates of size were obtained directly proportional to the sizes of the individual canals. Statistical analysis of these figures shows that there is a definite and significant difference between treated and untreated sides (the treated side

being greater), and therefore that increased blood-flow on the treated side has caused an increase of canal diameter through absorption of the wall. This is attributed to increased blood-flow and not to any increased activity of the osteoclasts, whose numbers do not show any change.

Leriche and Polcard's hypothesis, that increased vascularity of bone produces decalcification, is held to be proved, though the biochemical mechanism of this process remains obscure and offers further problems for investigation.

Dr BOURNE remarked that he was under the impression that the work of Leriche showed that if the bone of an animal was broken the phosphatase content of the whole skeleton increased and that this could mean either absorption or deposition of bone, since the enzyme is concerned in both processes. Dr FELL agreed that this was so.

Dr PRITCHARD said that he was surprised that the decalcification was not more extensive in view of the striking changes which may follow lesions involving the sympathetic in clinical practice. He suggested that the effects on bone of active and passive hyperaemia ought to be distinguished carefully as the former predisposed to calcification while the latter stimulated new bone formation.

Dr FIELD inquired if the sections were taken at comparable levels and whether they were cut at a slant. Dr FELL replied that the pieces from the right and left tibial shafts were taken from the same portions of the bones, and the serial sections selected for study from these pieces were from the same level. Great care was taken in all cases to get the plane of section at right angles to the long axis of the bone. He did not think that the amount of slant ever exceeded 5° and even that amount is readily seen in adjusting the block.

Dr F. FYFE inquired whether a planimeter had been used to measure the areas of the canals on a much enlarged image of the slides. Dr FELL replied that he had used this instrument but did not find it accurate enough, some of the smaller canals giving no reading.

Dr G. T. ASHLEY read a paper entitled 'On the insertion of the pectoralis major'.

Fourteen cadavers and four foetuses were examined to determine (a) whether the tendon is bilaminar or trilaminar, (b) which of the fibres actually twist before reaching their insertion, (c) through which lamina the various fibres are inserted.

In all cases the tendon was found to be bilaminar. This is contrary to the view expressed by Grant of Toronto.

The only fibres involved in twisting are the SUPERFICIAL fibres from the lower sternocostal and abdominal elements, plus, occasionally, a few superficial fibres from the manubrial and clavicular portions. These fibres join an aponeurosis on the inferior border of the muscle 1-2 in. from the bone before twisting backwards, upwards and then laterally, fanning out to form the posterior lamina of the tendon.

The anterior lamina is formed mainly of tendon derived from the manubrial portion. The majority of fibres of the clavicular portion are inserted into the front of this lamina, and a definite, though variable, contribution of deep fibres from the lateral edge of the body of the sternum and adjacent 3rd, 4th, 5th and (?) 6th costal cartilages passes without twisting to join the posterior aspect of the anterior lamina. These fibres are not described in the text-books.

From the sixth rib a bundle of non-twisting fibres passes upwards and laterally to join either the anterior aspect of the posterior lamina or the posterior aspect of the anterior lamina. These fibres are not described in the text-books.

The PRESIDENT suggested that the tendon of the muscle may be considered to be multilaminar some 2 in. from the line of insertion. Dr ASHLEY replied that the non-twisting part of the muscle is multilaminar at this point, but that as the muscle fibres become aponeurotic they fuse with those of adjacent bundles to form the accepted anterior and posterior laminae, and it is only rarely that multilamination of the tendon is apparent unless sharp-knife dissection is used.

Dr BRANDT raised a query on the analogy of the twisting of pectoralis major and the arrangement of the teres major and latissimus dorsi tendons. Dr ASHLEY replied that the question of twisting in other muscles was part of a wider problem under study.

Dr A. C. ALLISON read a paper on 'The histology of the stomach in the Insectivora'

In three unrelated Insectivora, *Elephantulus myurus*, *Suncus orangiae* and *Eremitalpa granti*, the stomach has been studied. While the gross structure differs greatly in these three genera, microscopical examination shows that the same type of glandular organization is present in all cases. This organization is simple and generalized. It is suggested that in the gastric glands there may be distinguished a gradient of organization which appears to be related to the degree of differentiation of the cells in the glandular epithelium.

Professors ZUCKERMAN and BOYD raised the question of the phylogeny of *Elephantulus*. Dr ALLISON replied that the family Macroscelididae, of which *Elephantulus* is a member, had been grouped by the older systematists in a suborder of the Insectivora, referred to as the Menotyphla, which exhibited distinct Primate affinities. Though later work had cast some doubt upon a close relationship between the Macroscelididae and the Primates, a recent detailed osteological study had reaffirmed such a relationship. Moreover, the presence of a menstrual cycle had been demonstrated in *Elephantulus*. Whatever the systematic position might be, the stomach in *Elephantulus* is very similar in form and relationships to the stomachs of the lower Primates.

To a question by Professor BOYD concerning the nature of the ectopic mucosa in Meckel's diverticulum, Dr ALLISON replied that as far as ectopic gastric mucosa in this situation was concerned, preparations examined personally had glandular tubules composed of mucoid cells, as in ectopic gastric mucosa in oesophageal 'erosions'.

Dr D. C. SINCLAIR read a paper on 'The intervertebral ligaments as a source of segmental pain'.

It has been maintained by Kellgren that the posterior intervertebral ligaments constitute an important source of referred pain in cases of sciatica and pain in the back. This contention is based on the results of injecting hypertonic saline in the region of the mid line of the back without verifying the position of the point of the needle radiographically. In this work, little attention was paid to the anatomy of the region into which the injections were made, and as a result it was assumed that the findings were due to the stimulation of the interspinous ligaments. These observations have been repeated under radiographic control, and the findings are inconsistent with the hypothesis that the resulting referred pain is due to the stimulation of the ligaments, while the evidence strongly suggests that it arises from the direct stimulation of nerve trunks in the vicinity, such as the medial branches of the posterior primary divisions of the spinal nerves. Accordingly, the significance of lesions of the posterior intervertebral ligaments in the production of segmental pain may be regarded as not yet established.

Dr WYBURN made a congratulatory comment on the fact that an alternative cause of low back pain had been suggested other than the inevitable prolapsed disc. He remarked also on the widespread nature of pain resulting from subcutaneous injections of hypertonic saline, from personal experience.

Professor BOYD, offering his congratulations, remarked on the excellent demonstration that Anatomy was still the basis for clinical science.

APRIL 1948

An ordinary meeting of the Society, for the Session 1947-48, was held on Friday, 30 April 1948, in the Rooms of the Zoological Society of London, Regent's Park, the President (Professor A. B. APPLETON) in the Chair.

Dr R. E. REWELL read a paper on 'The caecum of the Hyracoidea'.

The intestines of this Order were described by Owen (1832), Flower (1872) and Beddard (1908). The ileum ends in a dilated sac in the left iliac fossa. This is sacculated and two taeniae run, one on to the anterior and one on to the posterior surface from the colon. Each is associated with an artery which raises a small ridge of peritoneum. They are of

equal size. A straight length of gut with longitudinal muscle layer and about 20 cm long, runs from this sac and turns cephalad in the right iliac region. In *Dendrohyra* a small knuckle protrudes from this on the antimesenteric border, on to which a small artery runs on the posterior aspect. There is no anterior artery to it. I have not found this diverticulum in the genus *Procavia*. An anangious fold of peritoneum lies in the angle between the proximal dilated sac and the gut leading from it. Where this gut turns cephalad, two symmetrical diverticula arise from the antimesenteric border. They are relatively large, are directed caudad and end in a point. No peritoneal folds are associated with these and several small arteries run on to each from the mesentery without raising ridges.

Earlier authors considered the first sac to represent the true caecum, but Chalmers Mitchell (1916) favoured the paired diverticula and compared them to the structures seen in birds, in *Trichechus* and *Cyclopes*. Since the proximal sac lay on the left side and its anangious fold lay on the opposite side to the usual, he did not consider it to represent a caecum.

However, only the proximal sac has arteries on the morphologically dextral and sinistral aspects, while all caeca that I have examined have such, from lizards, such as *Varanus* and *Uromastyx*, up to the Primates. Moreover, this sac only is sacculated. On these grounds it may well represent the true caecum.

The PRESIDENT, congratulating the author, asked why the blood vascular pattern was stressed at the expense of other morphological features. Dr REWELL replied that this was deliberate and he wished to stress its importance in connection with the caecum of all mammals. He agreed with the President that it was not the sort of feature usually considered to be of importance, but he had found this distribution of arteries in all the mammalian, and even reptilian caeca examined. It is, for instance, universal throughout the Primates.

To Professor YOFFLY, who inquired as to the presence or absence of a caecal sphincter, Dr REWELL replied that the material was not well enough preserved for the histological study of this or any other feature.

Dr HINDLE asked if there was any peculiarity in the rodent caecum which might throw light on caecal morphology. Dr REWELL replied that he had examined a number of rodent caeca and they were all relatively simple and quite unlike that of the Hyracoidea, however complex their colons.

Dr G. H. BOURNE read a paper entitled 'Some observations on the mammalian adrenal gland'.

Over a period of some seventeen years histological examinations have been made of the adrenals of large numbers of Marsupials and other Mammals and a detailed list of the observations of other workers on the mammalian adrenal has been made. Details of the structure of the adrenals of some 250 species are thus available. The present paper consists of a summary of the main points which have emerged from this study.

These are

(1) The Monotreme adrenal differs appreciably from the adrenals of the Marsupials and the Eutheria and resembles those of the Reptilia.

(2) All Marsupials show an adrenal with the typical mammalian arrangement of cortex and medulla.

(3) There is a remarkable constancy of adrenal structure throughout the whole mammalian kingdom, from the lowest Marsupials up to Man.

(4) The cortical zones cannot be distinguished in the adrenals of all species.

(5) Lipid droplets are found almost invariably in some of the cells of the adrenal cortex of all species, including the Monotremes.

(6) Vitamin C (as demonstrated by the acetic-acid silver nitrate technique) appears to be present in high concentration in the adrenal cortex of Monotremes and some twenty-four other species of Mammals to which this test has been applied.

(7) A black pigment which appears to be melanin, and a yellow lipochrome pigment are present in the adrenals of a large number of species of Mammals.

(8) Some representative of the 'X' or juvenile zone appears to be present in the adrenals

of a large number of species of Mammals. First described by Howard-Müller in the mouse, representatives of the zone have been found in other rodents, Insectivores, Ungulates, eleven species of Marsupials, bats and one species of monkey.

Professor YOFFEY inquired if there was any species in which a constant amount of islet tissue occurred, if so, such might be useful in experimental studies. Dr BOURNE replied that it was not sufficiently constant in any one species to be significant. He agreed with a further comment by Professor YOFFEY that it would be useful to secure exact data on cortico-medullary ratios, but relatively few species had been studied in this respect.

Mr N. CAPENER read a paper on 'Physiological splintage'.

In the treatment of diseases and injuries of the locomotor system, support of joints in physiological positions is a recognized feature. Apparatus which will control the position, yet permit movement, and which may assist in the replacement of deficient muscles, is a well known method applied in the treatment of peripheral nerve injuries. Apparatus using springs and designed to facilitate the recovery of function by providing reflex stimulation through the proprioceptive system, has been advanced, particularly in the treatment of the hand, in the type of apparatus called 'Lively Splints' developed at Exeter.

Dr WHILLIS said that such splintage was a distinct advantage particularly in providing greater elasticity in central palmar control. In reply Mr CAPENER said that there was a common misconception that splints are devices to immobilize parts of the body, and that his main purpose was to interest members in a problem of applied anatomy and physiology in the hope that it would lead to co-operative effort by clinicians and anatomists to further study along these lines. The splints for the hand, while leaving free the greater part of the important tactile area do, by providing spring balance substitution for deficient muscles, aid in the normal activation of peripheral reflexes and diminish inhibitory activity which we may expect to arise in muscles ineffectively opposed.

Drs C. D. CHANNELL and J. WHILLIS presented a communication on 'The effect of ulnar nerve block at the level of the pisiform bone on movements of the ring finger'.

When the subject's metacarpophalangeal joint is fixed by the observer, extension of the interphalangeal joints is difficult and the subject complains of discomfort over the dorsum of the metacarpal head. The taut extensor tends to become displaced to one or other side due to the loss of the stabilizing effect of the muscles inserted into the extensor hood. The discomfort and loss of power can be explained on the assumption that the lumbrical does not produce the necessary relaxation of the flexor to allow the movement to occur.

When the first phalanx is allowed to move freely, and attempts at opening the hand are made, most of the power of the extensor is expended on the first phalanx which becomes hyper-extended owing to the loss of the stabilizing effect of the third lumbrical and the third palmar and fourth dorsal interosseus.

Attempts at the 'needle-threading' movement (flexion of the metacarpophalangeal and extension of the interphalangeal joints), using the thumb and the ring finger, result in ulnar deviation of the ring finger due to loss of the opposing action of the lumbrical. The metacarpophalangeal joint is hyper-extended, the first interphalangeal joint fully flexed and the second interphalangeal hyper-extended. The correct movement of the interphalangeal joints can be restored by passive flexion of the first phalanx.

Professor YOFFEY asked what the role of the first dorsal interosseous muscle was in gripping between the index finger and the thumb. Dr WHILLIS replied that with the fixed index it could not produce abduction but probably rotated the index and fixed the thumb metacarpal.

Dr J. J. PRITCHARD read a paper on 'Alkaline phosphatase in the uterine epithelium of the rat'.

The distribution of alkaline phosphatase in the uterine epithelium of the rat has been studied in the immature animal, during the stages of the oestrous cycle, in anoestrus, in

pregnancy and after bilateral ovariectomy, and following the administration of stilboestrol and progesterone

The findings suggest that oestrogens stimulate the manufacture of the enzyme and its secretion into the uterine lumen

Dr BOURNE remarked that since the production of glycogen is believed to follow the path of splitting off of phosphate from hexosediphosphate and subsequent condensation of hexose molecules, and since phosphatase appears to be an enzyme which could split off phosphate, it would be interesting to know whether Dr Pritchard had observed any parallelism between the distribution of glycogen and phosphatase in his preparations. Dr PRITCHARD replied that he had no glycogen preparations, and he thought that there was no evidence that phosphatase played any part in the synthesis of glycogen.

Dr FIELD remarked that there was no such parallelism in heart muscle, and added that here there was abundant glycogen and practically no phosphatase. Dr BOURNE added that phosphatase was not the only enzyme which could split hexosediphosphate.

Dr R. J. HARRISON read a paper entitled 'Observations on the ovary and reproductive tract of the Ca'ang whale, *Globicephala melaena*'

The material was obtained from a school of 300 Ca'ang whales killed at Torshavn, Faeroe Islands, in March 1947, and was made available through the kind offices of Dr Carl Bech, Chief Veterinary Officer, Faeroe Islands. Ovaries were removed from a total of thirty-eight females and entire reproductive tracts from twelve females and one 42 cm foetus. The specimens were obtained from six immature females, twelve maturing females, six females, whose ovaries contained one retrogressing corpus luteum, and fourteen adult females, the ovaries of which contained two or more retrogressing corpora lutea.

An account of the anatomy of the reproductive tract is given, and the general structure is shown to be essentially similar to that described for *Phocaena* by Meek (1918). The changes occurring in the ovaries, uterine tubes, uterine horns and uterus during growth are briefly described. A marked thecal gland is present around the actively growing follicles found in the ovaries removed from maturing whales.

The ovaries from five whales contained either recently ruptured follicles or developing corpora lutea. The histological appearances of these corpora lutea are described. Many of the markedly vacuolated luteal cells become multi-nucleated during the later stages. The old corpora lutea apparently persist for a longer time in the ovaries of *Globicephala* than in those of other Eutheria. However, the appearance indicates that the corpora lutea do eventually disappear, and do not persist throughout life in a degenerated state as is said to occur in the Balaenopteridae (Peters, 1939).

Dr OSMAN HILL commented on the curious evagination in the cervical canal and inquired if the author had studied the histology of this region. Dr HARRISON replied that this study was being undertaken, and Dr BAXTER remarked that it might throw light on the precise extent of cervix and vagina in these specimens.

Drs J. DAVIES and D. V. DAVIES read a paper on 'The mesonephros of the sheep'

The cranial third of the Wolffian body in the sheep is occupied by a peculiar glomerular structure called for convenience the 'giant glomerulus'. Here is a more or less continuous glomerular cavity, drained by about twenty tubules, and traversed by a series of fenestrated vascular plates. This giant glomerulus has been previously described briefly by Bremer in sheep, cow and deer, but he did not explain the manner of its development correctly. Caudally the giant glomerulus is in series, through a short transitional zone, with a zone of some forty or more ordinary Malpighian bodies similar to those found in other mammals. The study of the development of the various zones in the sheep's Wolffian body provides much information as to the factors determining the form and disposition of both the glomeruli and tubules in the vertebrates generally. Furthermore, the giant glomerulus provides a useful landmark for the study of the degeneration of the mesonephros and of the site of the urogenital union. No massive degeneration of the cranial end of the mesonephros occurs in the sheep and the urogenital union occurs through approximately the 6th-12th

tubules This is in contrast to the description by Felix of the human and accords well with that of most other workers on the mammalian mesonephros The cellular processes involved in the degeneration are similar in all mammals

Dr J DAVIES read a paper on 'The arterial blood supply of the Wolffian body in the sheep'

The arterial blood supply of the sheep mesonephros has been studied by means of injections with indian ink The vascularization of this organ is shown in relation, first, with the so called 'giant glomerulus' and secondly with the group of ordinary Malpighian bodies caudal to it The former comes to be supplied by a single large vessel from the aorta and appears in this light as an early functional unit The ordinary Malpighian bodies do not develop their connexions with the aorta until a relatively late stage, when the giant glomerulus is already beginning to degenerate MacCallum's findings (1902) of multiple efferent vessels in the glomeruli of the pig mesonephros is confirmed for the sheep, and the manner of their development and connexions is studied

Professor J P HILL congratulated the authors of the above two papers and commented on the differentiation of the glomerular capillaries in a site quite independent of the dorsal aorta This had been missed by the majority of previous workers

Drs G N C CRAWFORD and R BARER read a paper on 'The action of fixatives on living cells as studied by phase contrast microscopy'

The actions of various simple fixatives on living cells teased from the testis of *Salamandra maculosa* have been studied by phase contrast microscopy and photography This enables the cells to be examined during and after the process of fixation without further treatment or staining Other vertebrate cells in addition to those of salamanders were also studied but in less detail Of the substances studied, formalin has proved to be of particular interest The general actions are

(1) An initial shrinkage and distortion of the cell, varying in degree with the concentration used This is followed by

(2) A gradual re-expansion, lasting several minutes This may go on to

(3) Further swelling, with the exudation of 'bubbles' from the cell These bubbles can be made to swell or shrink by varying the osmotic pressure of the medium The swelling of the cell is very marked with concentrations of formalin below 7% The addition of saline reduces the degree of swelling but only to a minor extent The final appearance of the cell, even after bubbling has occurred, does not always greatly deviate from normal Nevertheless, the cell has undergone considerable structural and chemical alterations It is not certain how far these results apply to blocks of tissue in which mechanical conditions may prevent gross swelling and bubbling It seems probable, however, that the same underlying changes will occur

Dr BOURNE remarked that in view of the fact that it had been claimed that there is a decrease in the cytoplasmic contents of cells under different physiological conditions it would be interesting to know if any of these passed out into the exuded bubbles described

Professors XOFFEY and YOUNG inquired if the authors had made any measurements of cells fixed in formalin or formalin vapour with and without saline Dr CRAWFORD replied that while they had made no precise measurements it could be said that the presence of saline produced less swelling He considered that increased concentration of saline would most likely produce less shrinkage

Dr R BARER read a paper on 'The reflecting microscope in anatomical research'

The reflecting microscope which has been in use at Oxford for over a year is one constructed by Dr C R Burch, FRS It consists of a reflecting condenser and a reflecting objective, each composed of one large concave mirror and a small convex mirror The mirrors are made from speculum metal, the large mirrors being aspherical in order to correct for spherical aberration In its simplest form the microscope has a numerical aperture of 0.65, but this can be increased to 0.98 The great advantage of the reflecting microscope is that it is completely achromatic Once the object is focused in light of any colour the instrument is

in focus for *all* wave-lengths, from the far ultra-violet to the infra-red. This is particularly valuable for ultra-violet photography and especially for ultra-violet absorption spectroscopy of cellular constituents. Such work has hitherto been carried out using a quartz refracting microscope (Caspersson), a technique which necessitates the use of monochromatic light, and which is very laborious. With the reflecting microscope an image of the cell under study is projected across the slit of a spectrograph and a photograph is taken. If a line source (e.g. a mercury arc) is used, an image of that part of the cell lying across the slit is obtained *simultaneously* in all wave-lengths emitted by the source. The use of a continuous source of radiation, such as a hydrogen arc, enables a complete absorption curve to be obtained automatically by means of a recording microphotometer. Examples of the applications of these methods to the study of the structure and cyto-chemistry of various types of cell are discussed.

Another feature of unusual interest is the long working distance of the reflecting microscope. In the present model the distance between the lowest part of the objective and the object is 13 mm. This enables micromanipulation to be performed under magnifications of 600–700 times. The surfaces of organs *in situ* are now available for microdissection. High-power capillary and skin microscopy can also be carried out.

Professor YORRLEY inquired if the author had studied erythroblasts of marrow. Dr BARNER replied that he was actually working on this problem and on foetal blood cells.

Professor J. D. BOYD read a paper on 'Argentophil cells in foetal ectodermal epithelia'.

During observations on the development and nerve supply of the human external auditory meatus, dendritic cells, with a marked affinity for silver, were observed in the epithelial plug. These cells possess the morphological characteristics of microglia and of the resting phase of cells of the reticulo-endothelial system. They are believed to represent foetal stages of the dendritic cells (Langerhans' cells) of the adult skin. Such cells have also been found in numerous other epithelia of human and other mammalian material at the author's disposal. In the present communication their distribution in the cutaneous epithelia of the head region is described and their origin and possible significance discussed.

Dr BAXTER inquired if argentophil cells had been noted in epithelial cords not of ectodermal origin, such as the vaginal canal, and in which breakdown to form a lumen took place. Professor BOYD replied that this problem was being studied.

Dr BACSICH remarked that the cells described by Professor BOYD are known as the cells Langerhans to the dermatologists, who believe that they are either part of the macrophage system of migrating leucocytes. He had tried in vain to find an elective staining method for these cells. In Professor BOYD's specimens, as a by-product of a silver impregnation for neuro-histological purposes, these cells are represented with unsurpassed clarity, so that a successful approach to the study of their physiological and pathological behaviour is now possible.

To a question by Dr WEDDELL, Professor BOYD replied that these dendritic cells stain with methylene blue.

Drs P. BACSICH and G. M. WYBURN read a paper entitled 'Subcutaneous homo- and heterografts of the umbilical cord'.

Recent experiments (Bacsich & Wyburn, *Proc. Roy. Soc. Edinb.* 62, 1947) suggest that the sulphurated mucoproteins of cartilage and the cornea are responsible not only for the avascular nutrition and translucency (or transparency), but also for the constant success of these tissues as homografts.

Theoretical considerations led us to assume that the non-sulphurated and consequently more easily hydrolysable mucoproteins of the umbilical cord ground substance would allow survival only for a limited period by affording a somewhat reduced protection to the graft.

Results of present experiments on the fate of subcutaneous homo- and heterografts of umbilical cords of guinea-pigs seem to bear out this assumption.

The significance of these findings and the bearings of these results on skin homografts are discussed.

Dr G CAUSEY read a paper on 'The effect of pressure on nerve fibre size'

This work was undertaken to see if it is possible to show a centrifugal pressure along the axon of a myelinated nerve fibre

Circumferential pressure round the nerve to the medial head of the gastrocnemius of the rabbit was obtained by placing the nerve across a glass chamber filled with mercury, and sealed with a gel of sodium alginate at the openings through which the nerve was passed. The nerve was then fixed and sectioned. Measurements were made, on photographs of the sections, of the area of the whole nerve bundle and of the myelinated fibres.

Comparisons of sections above, at and below the site of pressure and of a control nerve from the opposite limb showed that for pressure of 80 mm Hg and over there was (a) a mean decrease of 25 % in the area of the nerve bundle at the site of pressure, (b) a decrease in the mean fibre diameter at the site of pressure, (c) an increase in the mean fibre diameter above and below the site of pressure, the increase above being significantly less than the increase below the site of pressure. This difference is in agreement with the existence of a centrifugal pressure along the axon.

In reply to Dr BOWDEN who inquired about the effect of blood vascular occlusion on the nerve fibres Dr CAUSEY replied that ligation of the artery and vein together did not block conduction, but application of pressure after such ligation would block conduction and alter fibre size.

Dr ROMANES asked if the thickness of the myelin sheath could be taken as a reliable guide, to which Dr CAUSEY replied that estimates of myelin thickness, apart from the total diameter, had not been made.

Dr J R HINSHAW (introduced by Dr G WEDDELL) read a paper on 'The pattern of innervation in the iris'.

The innervation of whole preparations of iris from white rabbits has been studied by the aid of the intravenous methylene-blue technique (Feindel, W H, Sinclair, D C & Weddell, G, *Brain*, 1947, 70, 495). Many marginal nerve trunks anastomose to form several concentric plexuses from which are derived networks of fine beaded fibres. These networks innervate both the internal and external surfaces and are found in relation to the circular muscle. Perivascular nerves are rarely seen in the iris and this is in contrast to the prominence of perivascular nerves observed in many other tissues prepared by this method. The interstitial cells are well stained and show no connexion with the nerve fibres.

The iris is an ideal structure for the experimental study of autonomic innervation and the normal pattern of innervation has been mapped out in detail as a basis for such an investigation.

Dr MEXLING commented that Dr HINSHAW's statement that the interstitial cells showed no connexion with the nerve fibres, could be due to a partial staining of the nervous structures. He had often observed this in his own preparations, but when these were well and totally stained a connexion with post-ganglionic fibres could always be made out. The nervous networks of beaded fibres demonstrated by Dr Hinshaw are probably the processes of anastomosing interstitial cells of which the nuclei are not stained. In reply Dr HINSHAW stated that he was sure that there was no connexion between the interstitial cells he had demonstrated and the nervous network.

Drs G WEDDELL, W H FEINDEL and D C SINCLAIR read a paper on 'Pain sensibility in deep somatic structures'.

The hypothesis of Lewis (1938, Suggestions relating to the study of somatic pain, *Brit med J* 1, 321) that the skin and the deeper somatic tissues are supplied by two separate systems of pain nerves has been examined in the light of the recent work of Weddell, Sinclair & Feindel (1948, An anatomical basis for alterations in the quality of pain sensibility, *J Neurophysiol* (in the Press)) who found that alterations in the quality of pain sensibility can be produced by alterations in the pattern of the pain producing impulses reaching the central nervous system. A preliminary examination of the innervation of

certain deep somatic structures has been made, and it has been found that this innervation differs both in pattern and in density from that of the skin. These histological findings are sufficient to explain the observed differences in the subjective and objective results of painful stimulation of the skin and of deeper lying structures respectively. There is thus no need to postulate two separate pain-conducting systems supplying somatic tissues.

Dr MEYLING remarked that the terminal nervous structures demonstrated are probably autonomic nerve fibres, because they exhibited the typical varicosities which are always seen in peripheral parts of the autonomic nervous system.

Drs E J FIELD and J B BRIERLEY read a paper on 'The anatomy of the spinal nerve root lymphatic drainage'.

That a functional communication exists between the spinal subarachnoid space and the prevertebral lymph nodes in the rabbit has been demonstrated in a previous communication to the Society. The precise course of the lymphatic vessels concerned could not, however, be determined by the method then adopted to show this connexion. It has now been possible by a method of retrograde filling of these lymphatics from the abdomen after ligation of the thoracic duct in the thorax, to demonstrate their course in full. Vessels which commence in the substance of the posterior spinal nerves pass ventrally and receive tributaries from the region of the nerve roots and from the interior of the vertebral body. Such lymphatics end in one of several longitudinal channels on the front of the spine. Along such channels are the small lymph nodes which constitute the 'regional' nodes of the spinal subarachnoid space. These vessels merit attention in any consideration of the route whereby infective agencies may extend from the lymphatic to the central nervous system, particularly as they do not possess valves. Their existence, moreover, may afford a plausible explanation of some of the puzzling results in recent experimental work on poliomyelitis.

Dr BOURNE inquired why the authors assumed that the carbon particles passed through the cytoplasm of the cells—it seemed more likely that they passed between the cells. Dr FIELD replied that various authors had claimed that the pathway was through the cytoplasm and not intercellularly, and they concurred with this view.

Professor BOYD remarked that there were Hyrtl preparations demonstrating lymphatics in relation to the meningeal sheaths of dorsal root ganglia in the museum of University College, London.

Dr P GLEES read a paper on 'The localization of efferents from Area 4 in the internal capsule and corpus callosum of the macaque'.

Following an experimental study of the distribution of the cortico-spinal fibres from Area 4, further experiments have been performed to study the localization of the cortico-spinal and other descending fibres from Area 4 in the internal capsule. There is, however, no definitive area within the internal capsule assigned to a particular subdivision of Area 4. On the contrary, the fibres from all subdivisions of Area 4 intermingle in their descending course. The callosal distribution of Area 4 and its subdivisions have been investigated as well.

Professor BOYD commented on the limitations of the pyramidal system in some animals, to which Dr GLEES replied that in those animals which have a small pyramidal tract, often ending at the cervical level, a similar comparatively small sensory cortical projection occurs, as had been shown recently by Adrian for the pig where only impulses from the snout region reach cortical level.

Professor YOUNG inquired about recovery rates following lesions, and as to whether there was any evidence that leg areas 'take over' from arm areas. Dr GLEES replied that as far as the monkey is concerned, fairly quick motor recovery results after small lesions in Area 4. He assumed that neighbouring parts of the motor area can 'take over'—e.g. the leg for the arm area. Certain anatomical arrangements favour this view, but more experimental evidence is required.

Dr J T ARKLE read a paper entitled 'Effect of peripheral connexions on the maturation of regenerating nerves'

Previous work has shown that the type of connexion made at the periphery by a regenerating nerve influences the degree of maturation. Experiments were performed in which a muscle nerve was allowed to regenerate along different distances during a 100 day period. It was found that the greater the distance traversed by the regenerating fibres, the larger they became. Motor fibres which had travelled a long distance in an adjacent sensory nerve became larger than those travelling a short distance. The effect was most marked where the sensory nerve was still connected to its peripheral end-organ system.

Other regenerating nerves were implanted into muscles, some already innervated and others denervated, and the degree of maturation assessed after a 100 day period. The nerve implanted in the paralysed muscle produced a much more completely regenerated nerve than did that of normal muscle on the other side. The implants into normal muscle, however, were more mature than those nerves ending blindly as a neuroma in the fascia or where the regenerating tract leads to the peripheral end organs of a sensory nerve.

It appears, therefore, that the process of maturation during regeneration is influenced by such factors as the distance the young fibres have to travel, and also the possibility of making functional motor endings on muscle fibres. Further experiments showed that fibres turn back at a neuroma, if the latter is removed, the number of fibres in the stretch of nerve above it decreases. The crowding produced by the recurrent fibre diminishes the size of all fibres in the nerve.

Dr ROUVES asked whether the turning back of the fibres at the neuroma would not of itself lengthen the pathway of regeneration, and Dr ARKLE pointed out that any such effect would be limited to the very small-sized fibres.

Dr BOWDEN stated that the organization of voluntary movement was related to the size of the nerve fibres, and commented on the effect of length as an added factor affecting the fibre diameter. On inquiry by Dr GLEES it was stated that the paralysed muscles responded to a smaller stimulation of the implanted nerve than did the normal muscle.

JUNE 1948

The Summer Meeting of the Society, for the Session 1947-48, was held in the Department of Anatomy, of the University of Bristol, on Friday and Saturday, 25 and 26 June 1948, the President (Professor A B APPLETON) in the Chair.

Drs J B BRIERLEY and E J FIELD and Professor J M YOFFEY read a paper entitled 'Passage of indian ink particles from the cranial subarachnoid space'.

It has been generally believed that particulate matter cannot, under normal pressure conditions, escape from the cranial subarachnoid space to the extracranial lymphatics. In experiments on rabbits, using a particle suspension of known size, ink was introduced into the cisterna magna, replacing an equivalent quantity of cerebrospinal fluid at a pressure of 120 mm of water. Within a few hours ink was found in the submucous lymphatics of the nose, having travelled along the subepineural space of olfactory nerve bundles and along intraneural clefts not lined by endothelium.

Dr R J HARRISON inquired if macrophages were present in the normal mucous membrane. Dr FIELD replied that some indian ink particles were within phagocytic cells while others were free. The main emphasis, however, was not on the segregation of indian ink particles by macrophages but on its presence in pathways leading from the cranial subarachnoid space.

Drs J B BRIERLEY and E J FIELD read a paper on 'The retro orbital tissues as a site of functional connexion between the subarachnoid space and the lymphatic system'.

The well known extension of the subarachnoid space around the optic nerve has been further investigated in the rabbit by the indian ink technique described in a previous

communication to the Society. Ink particles were seen to penetrate the optic-nerve sheath throughout its whole extent and coronal sections showed that their concentration decreased from the nerve towards the bony wall. The retractor bulbi muscle was heavily stained with ink, the particles lying in parallel columns between their fibres. The recti muscles were less intensively stained, but in some cases the ink tracked forwards to their scleral insertion. No true lymphatics were ever observed within the orbital cavity.

The diffuse spread of particles appeared to justify the injection of ink behind the globe in order to define the further course of fluid drainage. Twenty-four hours after injection ink was noted in the upper pole of the deep cervical lymph nodes. There was dense accumulation of ink around the nerves entering the orbital apex.

The suggestion is advanced that the cerebrospinal fluid contributes to the tissue fluid of the orbit, draining normally towards the apex of that cavity, where it is absorbed into lymphatic vessels which are afferents of the deep cervical lymph nodes.

Thus the retro-orbital tissues around the optic nerve cul-de-sac may stand in the same relation to the cervical lymphatic system as the epidural tissue of the spinal nerve roots to the pre-vertebral lymph nodes.

Dr WHILLIS commented on the relative size of the spaces around the optic and olfactory nerves and Dr BRIERLEY said that he could not offer any explanation for this difference.

To Professor KIRK who inquired about the relationship of Tenon's capsule to the sub-arachnoid space, Dr BRIERLEY replied that this space was very difficult to define in the rabbit and that the ink introduced was confined to the posterior surface of the bulb.

The PRESIDENT in thanking the authors said that he was pleased to see the expansion of the work in the lymphatic system begun by Professor Yoffey. The present findings, he thought, may prove to be significant in connexion with the mechanism of exophthalmic goitre.

Drs W. H. FEINDEL and A. C. ALLISON read a paper on 'Intravital methylene blue as a method for staining degenerating fibres in the central nervous system'.

An intravenous technique which permits a much wider application of methylene blue as a vital stain for nervous tissue (Feindel, Sinclair & Weddell, *Brain*, 70, 495, 1947) has been modified (Feindel & Allison, *Science*, 23 April 1948) to allow for a study of degenerating fibres in the brain. In rabbits perfused with methylene blue 5 days after enucleation of one eye, degenerating axons in the optic tract are rendered remarkably conspicuous by their intense and metachromatic staining as compared with normal fibres. In addition, morphological changes of early degeneration, such as fibrillation and vesiculation of the axon cylinder, are clearly demonstrated. The results are briefly described and compared with those obtained by the Marchi method and by metallic silver impregnation. It is likely that the method may find useful application for defining accurately the anatomical distribution of fibre degeneration produced by selective experimental lesions of the central nervous system.

Professor GOLDBY inquired if the technique had been applied to the peripheral nervous system. Dr FEINDEL replied that the method was not yet perfected, but it might well be extended to the peripheral nervous system. He further remarked that the results were rather empirical and that further work on the chemistry of the technique was in progress.

Mr RICHARDSON inquired if oxidation products might be liberated when this dyestuff was used, and was informed that further research on the chemistry of the technique together with spectroscopic investigations might elucidate this problem.

Dr H. A. MEYLING (introduced by Professor R. D. LOCKHART) read a paper entitled 'Structure and significance of the peripheral autonomic nervous system'.

To study the peripheral autonomic system both silver impregnation and vital methylene blue staining are used. Schabadasch's modification of the latter, specially valuable, demands very exact use. Dilute methylene blue (0.01%), buffered (pH 5-6) with $MgBr_2$ (1.25-2.0 g/l) and glucose (2.0 g/l), is either injected intra-arterially or the tissue is immersed in the solution for 10-20 min. The tissues are then left for 10-20 min in the solution without

methylene blue, fixed in 2.11 % ammonium iodide in saturated ammonium picrate and mounted in glycerine saturated with ammonium picrate. Precise timing and exact buffering, differing for each tissue, will stain totally the peripheral extension of the autonomic nervous system, while other tissues, especially connective tissues, are unstained. In all the tissues examined (conducting system of the heart, endocardium and pericardium, stomach, intestines, urinary bladder, gall bladder, small blood vessels, carotid sinus and aorta in the horse, cow, sheep and rat) a nervous network was found, consisting of autonomic interstitial cells of Cajal. As the post-ganglionic and efferent fibres are connected with this network, since Nissl substance is present in the vicinity of the nuclei, and since the processes of the interstitial cells show typical varicosities and, especially in silver preparations, a neurofibrillar structure, it is concluded that these cells are of nervous nature.

Most authors agree that autonomic fibres end as a network, containing nuclei. Since these nuclei are surrounded by a network of neurofibrils and these neurofibrils also form a fine network in the processes of the cells, these cannot be Schwann cells. Sympathetic and parasympathetic fibres are unlikely to form one network, which, persisting after the post-ganglionic and afferent fibres are cut, must be formed by the processes of the interstitial cells. In the different tissues this network differs only in the form of the pattern and the number of nuclei. An anatomical conception of a primitive intrinsic nervous network (similar to the sensory-motor network in invertebrates), upon which end both the post-ganglionic and afferent fibres, harmonizes with the physiology, explaining continuing local function after autonomic section and providing a basis for the view that adrenalin acts at the periphery upon some intermediary between post-ganglionic terminations and tissue cells.

Professor BOYD said that he considered the author's technical methods insufficient to permit accurate recognition of interstitial cells. He was doubtful if the plexus formation of interstitial cells of Cajal did in fact exist. Dr MEYLING replied that he had no doubt about the plexus ramification as a continuous network. He had employed dilute solutions of methylene blue, well buffered, and which could only give a selective staining.

Dr D. E. BOLT and Professor J. M. YOFFEY read a paper on 'Inability to detect macrophages in degenerating nerves.'

In a number of rats the sciatic nerve was divided and the animals subsequently killed at intervals ranging from 1 to 73 days. Although the cut ends were left free, in the experiments of longer duration some regeneration occurred. Animals were deeply stained *intravital* with trypan blue for 5 days before they were killed. No dye containing cells were ever found in the interior of a nerve bundle, though macrophages in the epineurium were heavily stained.

To Dr SANDERS, who inquired if there was any evidence of conversion of Schwann cells into macrophages as alleged by some authors, Professor YOFFEY replied that staining of Schwann cells by trypan blue was an infrequent occurrence—when they did so the nuclei showed quite a deep stain also.

Mr RICHARDSON suggested that owing to the interference with blood vessels of the nerve no dye got through, and consequently the macrophages, though they were there in abundance, could not be demonstrated simply because the dye did not reach them. Professor YOFFEY replied that there was ample evidence that the dye did get through and they hoped to present this evidence later. If anything, the permeability of the blood vessels in degenerating nerves was increased.

Dr E. L. PATTERSON read a paper on 'The arterial blood supply of the sympathetic trunks in man.'

A macroscopic survey of the arteries concerned in the blood supply of the sympathetic trunks has been made by injection of the arterial system of foetuses with indian ink. Ganglionic branches from the ascending pharyngeal and inferior thyroid arteries to the cervical trunks, from the superior intercostal, aortic intercostal and lumbar arteries to the thoracic and lumbar trunks, and from the lateral and middle sacral arteries to the sacral trunks, are described.

Professor KIRK congratulated the author on the painstaking and delicate dissection required to reveal the blood supply to these sites. He was surprised that the vertebral artery did not give a rich blood supply to the inferior cervical ganglion.

Dr MORTON inquired if there were any differences in the pattern of the blood supply at different age periods. Dr PATTERSON replied that the specimens dissected were all at or near full-term and that he had observed no significant differences in them.

Dr FEINDEL asked if the arteries to the sympathetic trunk were arranged in segmental fashion. Dr PATTERSON replied that in the thoracic and lumbar regions the arrangement was in general segmental, each ganglion receiving one or more branches from the corresponding segmental artery, but that not infrequently the ganglion received additional vessels from adjacent segmental arteries above or below its level.

Dr F. K. SANDERS read a paper on 'The fate of nerve homografts in the rabbit'.

Pieces of tibial or peroneal nerve 2-10 cm. long were taken from donor rabbits and used to fill gaps in the peroneal nerves of other rabbits, being fixed in place with clots of autogenous plasma. Grafts were removed and examined histologically at intervals from 2 days to 6 months after operation, their appearance being compared with that of control autografts placed on the opposite side of the same animal.

The Schwann cells of an autograft survive and multiply, and the myelin breaks up and is removed, so that in the later stages autografts are indistinguishable from normal peripheral stumps. The Schwann cells of homografts, however, undergo complete destruction within the first week after grafting, the graft at the same time being invaded by large numbers of lymphocytes, these being especially numerous in the region of the junctions. The collagenous framework of the graft, however, persists for a long time, being invaded by large numbers of macrophages, presumably to remove the debris of degenerated nerve fibres and destroyed Schwann cells. Regenerating nerve fibres, accompanied by Schwann cells, are able to invade such grafts from the central end. At the same time, Schwann cells from the host nerve migrate into the graft from its distal end, and when the graft is short (less than 3 cm.) the two streams of cells meet somewhere within the graft, giving such grafts at a late stage an appearance which does not differ strikingly from that of autografts. In long grafts (5 cm. and over), owing probably to a failure of vascularization, the two streams of cells fail to meet. As a result such grafts at a late stage show a proximal zone resembling a successfully reinnervated peripheral stump, separated from a distal zone resembling a degenerated but uninnervated distal stump by a dense collagenous plug.

The implication of these and other findings with reference to the mechanism of destruction of homografts was discussed.

To a question by Professor YORREY as to whether or not there was evidence of haematogenous migration of macrophages once vascularization had occurred, Dr SANDERS replied in the affirmative.

Dr E. P. SAMUEL read a paper on 'The innervation of the articular capsule of the knee joint'.

A detailed histological study of the innervation of the articular capsule of man and of the cat has been undertaken to provide an accurate anatomical basis for a further inquiry into the sensitivity of these structures. It was found that a large proportion of the medullated and non-medullated nerves which ramified in the joint capsule did so in company with the blood vessels. They were distributed in a plexiform manner through the fibrous capsular ligament and synovial membrane, and gave off branches which (a) innervated the blood vessels, (b) formed a true nerve plexus in the synovial membrane (this plexus received branches from the nerves which entered the joint tissues independently of the blood vessels), and (c) ended in various types of free nerve endings.

Some new light has been thrown on the nature of the specialized endings in the joint capsule. All the types of endings previously described have been seen while examining 200 feline and 20 human knee joints, and it is felt that previous differences of opinion regarding their existence have arisen because some former investigators examined too

small a series of specimens. The exact nature of the nerve fibres in the articular capsule has hitherto remained unsolved, and in an effort to acquire more precise knowledge the knee joints of twelve sympathectomized cats have been studied and compared with joints of normal animals. The conclusions reached were that, though a large component of the nerves in the joint capsule were of a sympathetic origin, the synovial membrane, contrary to the views of Gerneck (1932), Plasserman and Daubenspeck (1938), and others had a substantial somatic nerve supply.

Dr AITKEN remarked that careful histological technique must be employed to distinguish nerve fibres, unless continuity from larger fibres to smaller ones can be traced, then the results must remain inconclusive. Dr SAMUEL replied that he was convinced that his technique demonstrated nerve fibres only.

Drs M REISS, F E BADRICK and J M HALKERSTON (introduced by Professor J M YOFFEY) read a paper on 'The uptake of radioactive tracers by the pineal gland'.

In confirmation of Borell and Örstrom it was found that the pineal takes up per mg fresh weight considerably more P^{32} than any other organ of the body. Borell and Örstrom are inclined to ascribe, therefore, a special hormonal function to the pineal in phosphorus metabolism. This assumption was found to be incorrect, since also the iodine uptake (I^{131}) of the pineal is, with the exception of the thyroid, considerably higher than all the other organs.

It has, on the other hand, been found that P^{32} and I^{131} uptake of the pineal is still further increased after hypophysectomy. Some antagonistic action between pituitary and pineal concerning the uptake of radioactive tracers is therefore assumed.

Dr J HAMILTON asked whether the calcareous deposits in the pineal gland had any relationship to phosphorus uptake. Dr REISS replied that he did not know, but that this problem would be worth while investigating.

Dr JACOBY commented on the observed increase in cell content of the pineal following hypophysectomy, and inquired if there was any evidence of mitotic activity. Dr FIELD, replying for the authors, said that no mitosis had been observed, but that this need not be regarded as unusual, some rapidly growing embryonic tissues showed none.

Dr F B ROBINSON and Professor J M YOFFEY read a paper entitled 'Effects of cold and adrenaline on the suprarenal cortex of the adult male Wistar rat'.

Fifty adult male rats were exposed to cold at 0°C for times varying from 5 min. to 7 days. Forty-four rats were given single intraperitoneal injections of adrenaline and killed at times varying from 5 min. to 1 week afterwards. Six rats were given repeated injections of adrenaline three times a day for times ranging from 1 to 7 days. Studied by means of the Schultz and other histochemical reactions, the suprarenal cortex showed depletion commencing within 5-10 min. after exposure or injection. In general, there is a close similarity in the response of the cortex to adrenaline and cold.

Dr REISS remarked that the interesting changes reported in the cholesterol distribution in the adrenal might be due to the endogenous mobilization of corticotrophic hormone in the cold. It has been shown that the corticotrophic hormone considerably reduces the cholesterol content of the adrenals in a few hours after injection, the cholesterol content being restored or even increased 2-3 hr. afterwards. The reduction in cholesterol is probably connected with the increased cortin production which takes place when rats are kept in the cold. He thought it most interesting that the changes described by Dr Robinson occurred mainly in the zona fasciculata and reticularis, while the zona glomerulosa remained unaltered. This may be in agreement with the assumption that the production of corticosterone (responsible for carbohydrate metabolism, etc.) takes place in the zona fasciculata, which also atrophies after hypophysectomy, while the production of desoxycorticosterone (responsible only for water and salt metabolism) in the zona glomerulosa is apparently not influenced by the pituitary anterior lobe.

Dr J D GREEN read a paper on 'The comparative anatomy of the vessels and nerves of the pituitary body'

The blood supply and innervation of the hypophysis has been studied in thirty-eight representatives of existing classes of vertebrate. With some reservations for Agnatha and Chondrichthyes, pending further study, the following generalizations appear to hold

(1) The derivatives of Rathke's pocket obtain their blood supply from the vascular mesenchyme, which at first separates them from the diencephalon

(2) The venous effluent passes caudally and laterally into the vascular plexus of the saccus endolymphaticus in amphibians or into the cavernous sinus in higher forms

(3) A hypophysio-portal circulation is developed in *Salientia*, is constant in higher forms and is characterized by a secondary vascularization of the median eminence of the tuber cinereum (found also in some caudata) and by collection of the effluent blood into portal channels which then supply the adenohypophysis

(4) In *Necturus* the dorso-caudal part of the infundibulum receives an independent blood supply. Progressive enlargement and specialization of this region, apparently paralleling the first acquirement of a land habitat, leads to the neural lobe of higher forms

(5) The histology of the neural lobe is suggestive of direct secretion from nerve terminals rather than through the pituicytes

(6) Endings are often seen in the pars intermedia. Vasomotor-like endings are frequent in the median eminence and rather rare in the pars tuberalis, but never in the pars distalis are nervous structures seen, though reticulum may closely simulate them

Dr WALLS suggested that a radio-opaque substance such as thorotrast might be employed to demonstrate the course of the circulation. This would provide a permanent photographic record of the direction of the blood flow. Dr GREEN replied that the direction of blood flow had been observed directly by Houssay *et al*, and by himself in a variety of Amphibia. He agreed that it was desirable to show this in higher vertebrates, but he doubted whether the method suggested was as reliable as actual observation of the living circulation

Dr G W HARRIS asked why the author stated that the median eminence of the neurohypophysis contained relatively few nerve fibres as compared with the infundibular process. Dr GREEN replied that the fibres which passed through the median eminence branched when they reached the infundibular process, giving the appearance of more nerve fibres in the latter

Dr G W HARRIS read a paper on 'Ovulation in the rabbit'

A technique has been developed for stimulating the basal areas of the brain in a group of conscious rabbits simultaneously and for prolonged periods. By this method it has been found that stimulation of the tuber cinereum in the unanaesthetized rabbit, for as short a period as 3 min, may be followed by a full ovulatory response. Direct stimulation of the anterior pituitary, pars intermedia or pituitary stalk, for periods up to 7½ hr was not followed by ovulation. These results are discussed in relation to the possibility of neurovascular transmission of stimuli from the hypothalamus to the anterior lobe of the pituitary via the hypophysial portal vessels

Dr JACOBY inquired if the author had any idea of the nature of the stimuli passing along the blood vessels. Dr HARRIS replied that there was some evidence for acetylcholine as a possible hormonal transmitter of the stimuli down the hypophysial portal vessels, though the work of Taubenhaus and Soskin, on which this hypothesis is based, was performed on the pseudopregnancy response in rats, and could be questioned on that ground. There was other evidence put forward by Markee, Sawyer and Hollingshead that some adrenaline like substance might be concerned, for these workers injected adrenaline directly into the pituitary gland in rabbits and obtained ovulation. But this evidence is also open to doubt, for the injection of any substance directly into the pituitary will result in the destruction of some glandular tissue, and possibly in the liberation of stored hormone into the blood stream

Dr J S BAXTER read a paper on 'Alkaline phosphatase activity in the renal tubules'. In the post-natal kidney of the rat, alkaline phosphatase is present only in those proximal

convoluted tubules which have previously been shown (Baxter & Yoffey—in the Press) to be functional as judged by their ability to store trypan blue. The enzyme is absent, or present in negligible traces, in the mesonephros of this form. It appears in the proximal segments of the metanephric tubules relatively late in pre-natal life and its activity is not very great when compared with the kidney after birth.

In the sheep, on the other hand, enzyme activity is marked in the mesonephros at the earliest stages examined (7.8 mm), and continues until well after the time when it is first seen in metanephric tubules (29 mm). Its activity in the latter is well marked during the remainder of pre-natal life.

Since the presence of the enzyme probably denotes a functional maturity of the mesonephric and metanephric tubules, the differences between the results in the rat and the sheep are probably to be correlated with the type of placentation in each.

Alkaline phosphatase is also present in the 'secretory' segment of the pronephric tubule in the tadpole stage of the frog and in fishes shortly after hatching. It would appear, then, to be an important substance in the vertebrate nephron.

Professor HAMILTON inquired concerning the state of affairs before the link up of secretory and collecting tubules during development. Dr BAXTER replied that alkaline phosphatase did not appear as a cytoplasmic enzyme until the tubules became structurally and functionally differentiated.

Professor J. D. BOYD read a paper on 'Argentaffin dendritic cells in urogenital epithelium of male mammalian embryos'.

Silver impregnated material of rabbit, pig and human embryos and foetuses shows the presence of an extensive system of dendritic cells in the epithelium of the prostatic and penile portions of the urethra, in the epithelial fusion between the prepuce and the glans penis and in related ducts. The dendritic cells are similar in morphology to those found in embryonic epithelium of the head and to the dendritic cells ('melanoblasts') of post-natal skin. Their distribution and possible significance are discussed.

Professor YOFFEY inquired if the processes of these cells actually enter the substance of neighbouring cells. Professor BOYD replied in the negative and added that in skin, cells of similar character 'pay off' melanin into epithelial cells.

Dr C. C. MACKLIN's communication entitled 'Mapping the epithelium of the finer bronchioles by supravital silverization' was read by Professor W. J. HAMILTON.

The fresh collapsed lungs of normal adult white mice were filled with ammoniacal silver solution, quickly drained and at once refilled with 10% neutral formalin. Strips of terminal bronchiolar epithelium, seen *en face* in flattened cleared frozen sections, were mottled, with irregular rows or islands of dark golden brown intermingled with clear tracts of analogous disposition. These sharply contrasting areas marked the air-presenting faces of two distinct and well differentiated cell types composing the bronchiolar epithelium, respectively the smaller silverized or dark, and the larger unsilverized, light or clear cells.

The faces of the dark cells were made up mainly of fine brown, silverized granules of uniform size and distribution, and their clear-cut borders were featured by points, joined by dark silvered lines, often concave outwardly. Towards the alveolated air-ducts these faces became narrow, relatively inconspicuous, and insunken between the protruding clear cells. In lateral views afforded by unstained paraffin sections the silverized cells relatively remote from the alveoli were quadrangular, with the faces underlying the cilia appearing as rod-like lines of golden brown in which two layers of granules could sometimes be discerned, while the incurved side walls and flat bases were represented by sheets of fine black granules. Distally these cells frequently appeared as sharply limned truncated pyramids with flaring bases and narrow brown apical surfaces at the bottoms of funnel-like depressions. They may be represented on the alveolar walls by the epicytes.

The clear cells, everywhere unciliated, formed islands and rows interwinding among the silverized cells. Peripherally they increased in collective area relatively to the dark cells.

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Mr F R WILDE read a paper on 'The nerve supply of the iliac vessels in man'

The general pattern of innervation of the arteries to the extremities is well known. The precise origin and number of such nerves, their ultimate source, and the degree of individual variation still await clarification. Moreover, the nerve supply of veins, regarding which anatomical literature is scanty, has assumed clinical importance of recent years.

The present communication summarizes the conclusions reached from dissection of the vascular nerves that supply the common and external iliac vessels, in full-term human foetuses.

Dr N TERESCHENKO expressed admiration of Mr Wilde's skill as a dissector, and remarked that he had worked with a French surgeon who considered that periarterial sympathectomy was invariably successful. In the case of the femoral artery special attention to two points was necessary, viz (1) that the first inch of the profunda must be carefully stripped as well as the femoral artery itself, as the nerve fibres following the profunda later returned to the main femoral trunk, and (2) that the nerve fibres travel in the superficial layer of the intima as well as in the adventitia and must be destroyed to ensure success, accordingly the denuded parts of the arteries should be painted with a carbolic solution which can be washed away after a few minutes.

Dr F W FYFE presented a communication entitled 'Some features of the interlocking pattern at the upper epiphyseal surface of the femur with reference to "slipped epiphysis"'

The interlocking of elevations and depressions on the opposed metaphyseal surfaces of the head and neck of the femur is not mentioned in the masterly paper of Key (1926), where the opinions of 102 authors are discussed. Nor is it cited in available papers from 1926 to the present, the most recent anatomical experiment reported being by Ollerenshaw and Wood Jones, whose attempts to force off the epiphysis in two normal femora aged 14 and 15 years, removed post mortem, caused fracture distally in the neck.

Key emphasizes the strength in children of the neck periosteum, which in adolescence atrophies towards the adult type. The epiphyseal junction is a curved surface, like the spherical head, but flatter centrally. If the periosteum were not tightly attached, angular stresses would tend to dislodge the neck from the cupped head.

The upper end of the femoral neck of a boy, whose epiphysis looks normal but had been loosened by bomb blast, shows a tendency for antero-posterior slipping after bisection coronally, periosteal support thus being reduced. This tendency is due to ridges running antero-posteriorly. Other femora show the elevations (papillae and ridges) in a variety of patterns. One type noted has the ridges running from a flat or depressed centre to the periphery, an arrangement capable of opposing stresses from all directions. Another type has a more definite arrangement, with antero-posterior ridges at anterior and/or posterior quadrants. All have areas with papillae scattered indiscriminately, mostly in the medial and lateral quadrants (the condition throughout the flat, horizontal epiphysis of the elephant), but these papillae are often low and rounded in contour. This series is at present too small for categorical statements, but since it is improbable that early enough cases will ever be examined at biopsy or post-mortem, it may help to explain the clinical fact that trivial injuries, harmless if sustained as stresses in some directions, are, nevertheless, the only etiological factors in 30% of cases (endocrine imbalance being quoted as the cause in the rest), and if the shearing stress is sustained in an antero-posterior plane by a femur whose epiphyseal ridges run in a similar direction, the head may be the more easily dislodged, other factors being equal, e.g. periosteal strength and cartilage-to-bone cohesion.

Professor MITCHELL said that the existence of papilliform elevations and reciprocal depressions interlocking like those of dermis and epidermis, was recognized by many orthopaedists as a stabilizing mechanism at epiphyseal junctions. Dr Fyfe's suggestion that these might be definite papillary epiphyseal patterns was novel and interesting, and it was conceivable that certain patterns might prove less efficient in resisting either shearing or angular stresses and so favour diastasis. If further investigations confirmed this suggestion, the matter would be of considerable interest to orthopaedic surgeons.

Professor YOFFEY suggested that a similar study might be made at other sites, e.g. at the

HISTOCHEMICAL CHANGES IN THE SUPRARENAL GLAND OF THE ADULT MALE RAT

BY J M YOFFEY AND J S BAXTER*

From the Department of Anatomy, The University, Bristol

The object of the present work has been to observe the changes occurring in the suprarenal gland of the adult male rat after the administration of pituitary adrenotropic hormone (PATH) and extract of suprarenal cortex (Eschatin) †

MATERIAL AND METHODS

Seventy-eight adult male rats of the Wistar strain were used, of which forty-six were treated, twenty were untreated and twelve were controls. In experiments lasting longer than 6 hr, PATH and cortical extract were injected subcutaneously. In experiments lasting 6 hr or less, it was thought desirable to have a precise starting-point, and injections were made into the femoral vein under brief ether anaesthesia, thus obviating a possible source of confusion due to varying rates of absorption after subcutaneous administration. It had been hoped that with brief anaesthesia (10 min) the adaptational reaction noted by Selye (1937) would be of only moderate intensity, and that additional changes would follow the administration of PATH. However, the interpretation of these further changes proved difficult. Six control rats anaesthetized for 10 min each, and then killed in pairs 1, 3 and 6 hr later, all showed a characteristic adaptation ('alarm') syndrome. Where the survival period was longer than 6 hr, and the injection was made without anaesthesia, the changes in the suprarenal cortex could be interpreted as an uncomplicated response to PATH or cortical extract. In six control rats which were given subcutaneous injections of normal saline, and killed in pairs 3, 6 and 12 hr later, no significant changes were noted in the cortex.

Dose and duration of experiment PATH was given to each of twenty-eight rats in a single dose of two sudanophobic units (Reiss, Balint, Oestreicher & Aronson, 1936) per 100 g body weight, and the animals were killed from 1 hr to 3 days after injection. Eschatin was injected daily into fourteen rats, in a single dose of 5 dog units (Harrop, Pfiffner, Weinstein & Swingle, 1932) per 100 g body weight, for times ranging from 1 to 14 days and killed 24 hr after the last injection. Four further rats were given a daily injection of eschatin for 3 days, and killed 12 hr after a single injection of PATH on the 4th day.

HISTOCHEMICAL TECHNIQUE

The animals having been killed by coal gas, the suprarenals were removed immediately and fixed in formol-calcium (10 % formol in distilled water, with the addition of 1 % calcium chloride) for 3 days, and washed in running tap water for 24 hr, they were then placed in a 25 % solution of gelatin (with a trace of phenol) in a desiccator, and

* Aided by a grant from the Colston Research Fund

† For supplies of adrenotropic hormone (Cortrophin Organon) we are indebted to Organon Laboratories Ltd. Extract of suprarenal cortex (Eschatin) has been provided by the courtesy of Dr Stanley White Parke, Davis and Co.

left in an incubator at 37° C for 30 hr After this embedding in gelatin, the block was placed in 10 % formol-calcium with cadmium chloride (1 %) added, and kept in the refrigerator at about 2° C for 3 days before cutting This technique produces a block with about the consistency of celloidin whatever the external temperature Sections were cut at 10 μ and mounted on gelatin-covered slides The following stains were employed, in the manner described

Sudan black Sections were rinsed for a few seconds in 50 % and 70 % alcohol, then stained for 3 min in a saturated solution of Sudan black (Gurr) in 70 % alcohol They were then washed in two changes of 50 % alcohol and mounted in glycerine jelly The technique for staining, as also for fixation and embedding, is substantially that employed by Baker (1945)

Phenylhydrazine At first 2,4-dinitrophenylhydrazine sulphate was employed (see Yoffey & Baxter, 1948), but it was found not to be so precise as 2,4-dinitrophenylhydrazine hydrochloride, and was therefore abandoned in favour of the latter Sections were treated for 3–4 hr in a saturated solution of 2,4-dinitrophenylhydrazine hydrochloride in equal volumes of 2 N/HCl and absolute alcohol They were then washed in two changes of a fluid consisting of equal parts of absolute alcohol and concentrated hydrochloric acid, allowing 5 min in each, and mounted in glycerine jelly The employment of hydrochloric acid was suggested to us by Prof Wilson Baker, in order to dissolve out 2,4-dinitrophenylhydrazine hydrochloride and leave only the 2,4-dinitrophenylhydrazones

Plasmal reaction Sections were well washed in running tap water and then in distilled water They were treated for 3 min with a saturated solution of mercuric chloride in water, after which they were immersed in Schiff's reagent (fuchsin-sulphurous acid) for 15 min After three washings in sulphurous acid water, each lasting 30 sec, they were washed in distilled water and mounted in glycerine jelly

Schultz reaction Sections were treated for 2 days with 2.5 % aqueous iron alum at 37° C They were then allowed almost to dry on the slide, and covered with a drop of equal parts of concentrated sulphuric acid and acetic anhydride, the cover-slip being sealed with paraffin wax The sealing is important, for it enables the specimen to be kept for 24–48 hr whereas if not sealed it becomes useless after a few hours

When weather permitted, sections after treatment with iron alum were exposed to bright sunshine for 3 days, as recommended by Schultz (1924) The combination of iron alum and sunlight was found to give more effective coloration than either alone

THE SIGNIFICANCE OF THE HISTOCHEMICAL REACTIONS EMPLOYED

The phenylhydrazine reaction The phenylhydrazine reaction was first applied to the suprarenals by Bennett (1940), who believed that it was a specific test for corticosterones Using phenylhydrazine hydrochloride, he worked with thick sections, and observed the reaction with incident light The specificity of the reaction was questioned by Gomori (1942), Albert & Leblond (1946) and others They maintained that the substances which stained with phenylhydrazine were the same as those which gave the plasmal reaction, and that the phenylhydrazine reaction gave no additional information

The plasmal reaction The plasmal reaction was first used by Feulgen & Voit (1924) and has been extensively employed in recent years. According to Thannhauser & Schmidt (1946), the reaction indicates acetals of fatty aldehydes (chiefly stearic and palmitic) bound with colamine glycerophosphate 'Plasmalogen', the substance which becomes transformed into 'plasmal' to give the reaction, is associated in the tissues with phospholipids, but not infrequently the extent of the area staining with Sudan black differs markedly from that staining with Schiff's reagent.

In general it is true that tissues—including fat—which give a positive plasmal reaction also give a positive phenylhydrazine reaction, though in the case of the suprarenal cortex the plasmal and phenylhydrazine areas may differ markedly in extent. Whatever the precise nature of the substance or substances stained by the plasmal and phenylhydrazine reactions, their distribution seems to vary in accordance with functional changes in the suprarenal gland in a manner which may throw some light on the gland's function.

The Schultz reaction The Schultz reaction was introduced in 1924, as an application to sections of the Liebermann-Burchardt colour test for sterols (see Fieser, 1936). According to Schultz the cholesterol has first to be oxidized by sunlight or iron alum to 'oxycholesterol', concerning the chemical nature of which Windaus, when consulted by Schultz (1924), was somewhat guarded, but which presumably corresponds to the dihydroxycholesterol of our terminology. According to Fieser (1936) 'the colour reactions distinguish sharply the unsaturated from the saturated sterols', and the question which obviously arises is whether the Schultz reaction is specific for cholesterol, or whether it is given by any other unsaturated sterols. On comparing the Schultz coloration with the colour reactions of some pure steroid hormones, it was noted that, whereas the Schultz reaction gives a green colour, progesterone crystals on a slide gave a bright yellow when treated with the Schultz reagents, as did oestrone, desoxycorticosterone acetate (Doca) gave a dull brick with a slight bluish tinge, and even androsterone, which is saturated except for the carbonyl group, became a brownish yellow.

Recently Everett (1947) has concluded that the typical Schultz colour response is limited to the diols formed from cholesterol by mild oxidation procedures. Everett further noted that when cholesterol was mixed in gelatin with lecithin and other fats, the Schultz reaction ceased to be positive at a cholesterol concentration lower than 1/10. The reaction, with a little care, is one of considerable precision. Even within a single cell it can be shown that its distribution is not uniform, for it is limited to the cytoplasm and does not involve the nucleus (Pl. 1, fig. 5). This being the case, it appears certain that the occurrence in the gland as a whole of Schultz-positive and Schultz-negative zones accurately reflects the distribution of the cholesterol derivatives which give the reaction.

The observation of changes in the distribution of cholesterol within the suprarenal gland is of considerable interest. It has been known for some time (see review by Tepperman, Engel & Long, 1943) that, after feeding diets rich in cholesterol, the gland may show a marked increase in its cholesterol content and even undergo hypertrophy. Recent work (Bloch, 1945, Sayers, Sayers, Long & Long, 1946) indicates that cholesterol accumulates in the suprarenals as the raw material out of which steroid hormones are made. It would not be unreasonable, then, to expect

that marked variation in the secretory activity of the suprarenal cortex should be accompanied by appreciable changes in its cholesterol content on a scale sufficient to allow of histochemical demonstration

In general distribution and intensity, the Schultz, phenylhydrazine and plasmal reactions, and the Sudan black stain, show a rough parallelism. As far as concerns the Schultz reaction and sudanophile lipoids, this is in accord with the view that birefringent crystals in the suprarenal cortex are composed of cholesterol, for Yoffey & Baxter (1948) noted that these crystals, though not themselves sudanophile are most abundant in the region of maximal sudanophilia. The correspondence of the plasmal and sudanophile zones fits in with the view of Thannhauser & Schmidt (1946) concerning the fatty nature of plasmalogen. Phenylhydrazine no doubt does form phenylhydrazones with cortical steroids, and possibly with the fatty aldehydes which yield the plasmal reaction. In the present work it has been found to stain lipoids generally in spite of attempts to dissolve out excess phenylhydrazine by hydrochloric acid.

It must, however, be noted that the plasmal and Sudan black zones do not always correspond, nor do the plasmal and phenylhydrazine zones. In the present state of our knowledge it is only possible to draw attention to these facts without being able to explain them adequately. With regard to the plasmal and Sudan black zones, several experiments showed zones which stained with Sudan black but did not give a plasmal reaction. Presumably the fatty aldehydes can disappear as a result of cortical stimulation while sudanophile lipoids are still present.

Pl 1, figs 9 and 10 illustrate a gland in which the phenylhydrazine and plasmal zones were markedly different, the former being much more extensive than the latter, and occupying most of the depth of the zona reticularis. Whatever the detailed chemical explanation, such experiments indicate that the criticisms of the phenylhydrazine reaction advanced by Gomori (1942) and others, at any rate in so far as it was equated with the plasmal reaction, are not altogether valid. In a personal communication sent to us immediately before publication, Dr J. Danielli reports a recent conclusion by Boscott, Mandl, Danielli & Shoppee (1948, unpublished), that 'the aldehyde reactions obtained by using the plasmal technique on the suprarenal cortex show the presence of some compound other than desoxycorticosterone or allied ketones'. With this the present experiments are in full agreement, for we find that, in different functional states of the cortex, either the plasmal-positive zone may sometimes be markedly greater than that which gives a phenylhydrazine reaction, or vice versa.

RESULTS

A THE NORMAL SUPRARENAL

In the suprarenal of the normal male rat the plasmal, phenylhydrazine, Sudan black, and Schultz colorations extend through the zona fasciculata and reticularis (Pl 1, figs 1-5) as far as the medulla, from which they are absent, except for nerve fibres and islets of cortical tissue which may sometimes be found in the medulla. The stain is usually strongest in the outer part of the zona fasciculata, and less dense in the reticularis, giving rise to a characteristic gradient. As a rule the transition between fasciculata and reticularis is not abrupt, except perhaps occasionally in the case of the plasmal and phenylhydrazine reactions.

The zona glomerulosa is not always well marked, though when it is present it may be the most heavily stained part of the cortex, with a clear subglomerular zone between it and the outer part of the zona fasciculata. The marked variability of the zona glomerulosa as compared with the remainder of the cortex would accord with the views of Greep & Deane (1947), in so far as they suggest different functions for glomerulosa and fasciculata. In view of this variability it becomes difficult to define what is a normal glomerulosa. Frequently the distribution of staining material is patchy, occasionally a varying length of glomerulosa may be clear and free from stain, while the remainder may be fully stained. In view of the possibility that the conditions of cage life (three to six rats in a cage) might repeatedly stimulate cortical secretion, eliciting at intervals Selye's (1937, 1946) alarm reaction, it was decided to try the effect of isolation. Three rats were kept in separate cages for 24 hr., and then killed, the suprarenals of these rats showed in two cases full staining of the glomerulosa, in the third case almost complete absence of staining. Three further rats were then isolated for 40 days, and precisely the same result was obtained, namely two with full glomerulosal staining, one with complete absence of staining. It seems reasonable then to infer that glomerulosal discharge (of material stainable with the plasmal, phenylhydrazine, Schultz and Sudan black reactions) whatever its cause is not a stress reaction. The zona fasciculata and zona reticularis, on the other hand, under the usual conditions of cage life, never show a discharge, though they can be made to do so in response to a variety of stimuli. Because of the variability of the glomerulosal staining reactions, even in the normal animal, attention in the present paper has been directed mainly to the zona fasciculata and reticularis, and only incidental observations have been made on the glomerulosa.

B THE EFFECT OF PITUITARY ADRENOTROPIC HORMONE (PATH)

(1) *Schiff's reagent, Phenylhydrazine, and Sudan black*

As already noted the results from 1-6 hr. after injection are complicated by the fact that these animals were subjected to brief ether anaesthesia. The effects of anaesthesia are being investigated in further detail, and will not be described here. With this qualification, the changes after PATH injection (plasmal, phenylhydrazine, Sudan black) may be summarized as follows.

One hour after injection Two experiments. In both there was depletion of the reticularis with all three reagents, while the zona fasciculata appeared normal.

Three hours after injection Two experiments. In both the staining of the outer fasciculata was intensified, but whereas in one the reticularis was depleted, in the other it was not.

Six hours after injection Five experiments. In two experiments the only obvious change was in the reticularis, which in one case showed slight weakening, in another marked depletion. In two experiments there was intensified staining of the fasciculata with weakening of the reticularis. The fifth experiment showed what we have termed a reversal effect, namely weakening of the fasciculata and intensified staining of the reticularis.

Twelve hours after injection Six experiments. Three appeared normal. One showed marked depletion of the reticularis, and between it and the fasciculata an

intermediate zone most evident with Sudan black. The remaining two showed weakening of the reticularis, with moderate intensification of the fasciculata in one of them.

Twenty-four hours after injection Six experiments. Two were practically normal. In one, depletion of the reticularis with phenylhydrazine and Sudan black, but not with Schiff's reagent. In one, a marked reversal effect with Schiff's reagent, but not with phenylhydrazine. One showed depletion of the reticularis with Schiff's reagent, but not with phenylhydrazine. One showed a slight reversal effect with Schiff's reagent.

Forty-eight hours after injection Five experiments. Two showed marked depletion of the reticularis with both the plasmal and phenylhydrazine reactions, and one of these (Pl 1, figs 9-11) showed a well-marked intermediate zone between fasciculata and reticularis, much wider with phenylhydrazine than with Schiff's reagent. Two were almost normal, though in one there was a narrow juxtamedullary zone completely depleted, and in the other a similar zone with weak staining. The appearances suggest that the stainable matter extends into the reticularis from the fasciculata. One showed intensification of the fasciculata as well as weakening of the reticularis.

Three days after injection Two experiments. One appears normal. One has an intensely stained narrow band in the fasciculata, and a large weakly staining reticularis.

(2) *The Schultz reaction after administration of PATH*

Six hours after the administration of PATH the Schultz reaction demonstrates marked changes (Pl 1, fig 6). It disappears altogether in the reticularis, and occupies only a narrow and weakly staining zone in the fasciculata. At 48 hr after injection (Pl 1, fig 12) cholesterol is present in amounts which are nearer the normal, though by the other tests employed the cortex is still far from normal.

Discussion of PATH results

Plasmal and phenylhydrazine zones do not always coincide There is frequently a difference in extent between the plasmal and phenylhydrazine zones. In two 48 hr experiments, for example, the reticularis showed marked depletion with Schiff's reagent, much less with phenylhydrazine (Pl 1, figs 9, 10). Cases also occur where the zone of phenylhydrazine depletion is wide, that of plasmal depletion narrow. It is important to compare sections taken through the middle of the gland, and to avoid oblique cuts through the cortex. In the present experiments the procedure adopted was to cut fifteen such sections at 10μ and discard the remainder of the gland.

Functional significance of plasmal and phenylhydrazine reactions That the substances which give the plasmal and phenylhydrazine reactions frequently do not coincide in extent would accord with the original interpretation placed upon the phenylhydrazine reaction by Bennett (1940). On the other hand, the fact that fat can give a well-marked phenylhydrazine coloration suggests some caution in accepting this interpretation. On the basis of the present series of experiments, all that can be legitimately inferred is that, in stimulation of the cortex by PATH,

characteristic changes are produced in both the plasmal and phenylhydrazine reactions, so that one can decide with some degree of certainty whether the cortex is in the resting state or not

Does a single dose of PATH induce cyclic changes? The fact that there can be fairly rapid depletion of the reticularis occurring within the first 6 hr, then seemingly normal cortex in some cases at 12 hr, with depletion again in evidence in some instances at 24 hr and at 48 hr, suggests the possibility that a single dose of PATH may produce cyclic changes, with repeated emptying and filling of the reticularis. This is obviously linked with a number of other questions the answers to which are not known, such as: How long can PATH remain unaltered in the blood? Can some of it be used to stimulate cortical secretion, while the remainder circulates unchanged in the blood to act on the cortex when the first wave of secretion is over? Is there a quantitative relationship between PATH and the suprarenal cortex? Recently introduced methods for the estimation of cortin in the blood (Hemphill & Reiss, 1947) may provide an important link in the chain of evidence. It is interesting to note that even 3 days after a single injection of PATH the cortex may not have returned to its resting state.

The functional zoning of the cortex. Arnold (1866) on morphological grounds first introduced the traditional zoning of the cortex into glomerulosa, fasciculata and reticularis. In the rat a narrow but definite subglomerular zone also exists. Bennett (1940) suggested in the cat a grouping into four zones—presecretory, secretory, postsecretory and senescent. In the rat, apart from the glomerulosa which would correspond to the presecretory zone of Bennett, the present experiments do indicate three further zones in the cortex, making a total of four zones in all. However, the zone which Bennett terms senescent appears to be one which may repeatedly fill with stainable matter and become depleted, so it may conceivably play a rather more active part in cortical functioning than the term 'senescent' would imply. If the stainable matter is associated with cortical hormones, then the zona reticularis could well be a storage zone for cortical secretion, which it might rapidly discharge when needed. If, on the other hand, the reticularis is a secretory zone, its cholesterol content would indicate much less secretory activity than in the fasciculata. In one of the rats isolated for 40 days there was marked increase of stainable matter in the reticularis, and this would agree with the idea that it was a storage zone in which secretion had accumulated under stress-free conditions. It should perhaps be noted that a division of the cortex into fixed zones may not quite correspond with the facts, since under varying functional conditions the cortex may show a good deal of plasticity. It may even be that the different zones are to a considerable extent interchangeable.

The outer part of the fasciculata, from its rich cholesterol content, would appear to be secretory. The reticularis may be either weakly secretory or a storage zone. The purpose of the intermediate zone is not quite clear, but from observing differences in its width, it seems conceivable that it may represent the gradual extension of stainable matter, possibly secretion, from the outer to the inner zone (Pl. 1, figs 9–11).

Variability of results. In spite of apparent uniformity in dosage and other experimental factors, the response of the suprarenal gland in the various animals was not

constant We are unable to account for this, and can only speculate that either the glands were not all in the same functional condition at the outset, or else that in some animals unappreciated differences in the conditions prevailing at the time of injection may have evoked additional responses, of Selye's (1946) adaptational type

Significance of cholesterol changes The diminution in the cholesterol content of the fasciculata may be attributed to its conversion into steroid hormones which are discharged into the blood stream, and presumably fresh cholesterol would be taken up from the blood by the cells of the cortex to replace that utilized in the elaboration of hormones If hormone secretion is stimulated, its rate of formation and discharge could conceivably exceed that of cholesterol replacement so that a very active cortex might be almost devoid of cholesterol This view is supported by the finding, already noted, that 6 hr after a single injection of PATH the cholesterol content of the cortex shows a marked diminution Conversely, if the discharge of hormone is retarded, the rate of cholesterol deposition in the cortex might either be unchanged, in which case it would gradually accumulate in the gland, or else the cholesterol would only be taken up as required for conversion into the hormone, in which case it would not show a great increase In order to determine whether the cholesterol taken up by the suprarenal cortex was (a) independent of, or (b) ran parallel with the secretion of steroid hormones, a number of rats were given repeated injections of cortical extract The effect of this would presumably be to make it unnecessary for the cortex to secrete its own hormones, and if (a) were true cholesterol would accumulate in the cortex, whereas if (b) were the case it would not

C THE EFFECT OF CORTICAL EXTRACT

The effect of repeated administration of cortical extract was to cause intensified staining by all the methods employed, in both the glomerulosa and the remainder of the cortex, during the first 3 days Subsequently there was a gradual weakening, which by 14 days was very marked The simplest explanation of this sequence of events is that for the first 3 days the gland was functioning normally, and that steroid hormones and their precursors accumulated in the cortex When the administration of cortical extract was continued beyond this stage, the cortical cells underwent degenerative changes The Schultz response at the end of 3 days is very heavy both in the glomerulosa and the fasciculata It never becomes very intense in the reticularis, though as it accumulates in the fasciculata the latter zone may encroach considerably on the former

D THE EFFECT OF CORTICAL EXTRACT FOLLOWED BY PATH

If cortical extract is given daily for 3 days, the accumulation of cholesterol is intense (Pl 1, fig 7) If then on the fourth day PATH is given, this accumulated cholesterol quickly disappears from the fasciculata and the reticularis, but not from the glomerulosa (Pl 1, fig 8) As far as the fasciculata is concerned, this finding fits in well with the view that cholesterol is one of the raw materials for the manufacture of cortical hormones The glomerulosa, on the other hand, does not respond in the same way to PATH It has already been noted, on the basis of observations on isolated animals, that depletion of the glomerulosa does not appear to be a stress

reaction, and the further observation that PATH does not influence its cholesterol content even when the latter is heavy seems to confirm this

SUMMARY AND CONCLUSIONS

1 The reactions of the suprarenal glands of adult male Wistar rats to pituitary adrenotropic hormone (PATH) and extract of suprarenal cortex have been studied, using Sudan black staining, as well as the plasmal, phenylhydrazine and Schultz reactions

2 Changes in the glomerulosa appear to be independent of those in the remainder of the cortex

3 The view is advanced that, while the fasciculata is secretory in function, the reticularis may be a storage zone

4 An intermediate zone between the fasciculata and reticularis is sometimes very evident

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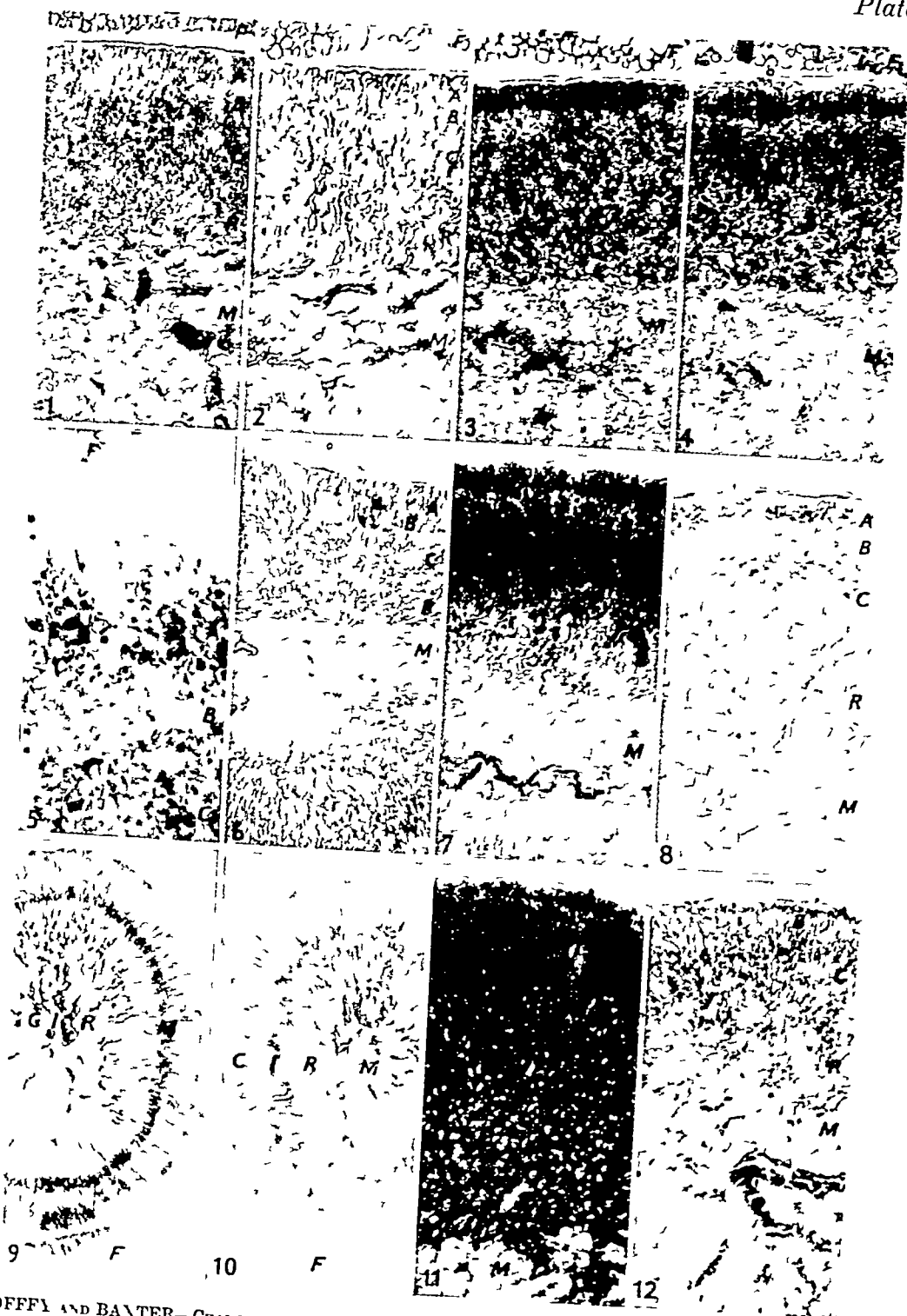
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EXPLANATION OF PLATE

All the illustrations are photomicrographs of frozen sections 10μ in thickness *A*=glomerulosa, *B*=subglomerular zone, *C*=fasciculata, *R*=reticularis, *M*=medulla, *I*=intermediate zone, *F*=Peri capsular fat

Note Some of the lettering is slightly out of place In Fig 1 the letter *B*, in Fig 3 the letters *A* and *B*, in Fig 6 the letter *A*, in Fig 7 the letters *A*, *B* and *C*, in Fig 8 the letters *B* and *C* all should be slightly nearer the surface of the gland, to indicate the layers correctly In Fig 9 the lettering should be reversed so as to form a mirror image of Fig 10

- Figs 1-4 Suprarenal of normal adult male rat, showing the plasmal (Fig 1) and phenylhydrazine (Fig 2) reactions, Sudan black stain (Fig 3), and the Schultz reaction (Fig 4) Note the glomerulosa and subglomerular zone, and the moderately marked gradient of staining in the remainder of the cortex, the staining being slightly more intense in the fasciculata, and weaker nearer the medulla There is no widespread staining of the medulla, though in Fig 4 the medulla has an islet of cortical tissue $\times 50$
- Fig 5 High-power view of part of Fig 3, showing the localization of the Schultz reaction to the cytoplasm of the cell, the nucleus being Schultz negative $\times 200$
- Fig 6 Diminution of the Schultz positive zone 6 hr after a single intravenous injection of PATH $\times 35$
- Fig 7 Schultz reaction in the suprarenal of a rat which had been given daily injections of cortical extract for 3 successive days, and was killed on the 4th Compare with the Schultz reaction in the normal gland (Fig 4), and note that the reaction is now much heavier both in glomerulosa and remainder of cortex, that the strongly Schultz positive zone is wider, extending well into the reticularis from the fasciculata $\times 50$
- Fig 8 Schultz reaction in the suprarenal of a rat which had been given daily injections of cortical extract for 3 successive days, then on the 4th received a single dose of PATH and was killed 12 hr later Compare with Fig 7, and note the marked diminution in cholesterol content of fasciculata and reticularis $\times 50$
- Figs 9-12 Suprarenal gland of rat killed 48 hr after a single injection of PATH Fig 9, plasmal reaction, Fig 10, phenylhydrazine reaction, Fig 11, Sudan black stain, Fig 12, Schultz reaction The photographs are from a set of fifteen sections taken through the middle of the gland The plasmal reaction is positive over a narrower zone than the phenylhydrazine Fig 9 shows a well marked intermediate zone, and Fig 10 a broadening of the intermediate zone so as to give a reversal of the usual colour gradient, with the reaction more intense in the reticularis than the fasciculata Fig 11 shows a positive Sudan black stain in a region which gives a negative plasmal and phenylhydrazine reaction (Figs 9 and 10, $\times 17.5$, Fig 11, $\times 50$, Fig 12, $\times 35$)



AN INJECTION APPARATUS INCORPORATING A MAXIMUM PRESSURE SAFETY DEVICE

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The use of liquid Latex or Vinylite Resin for making corrosion specimens is accompanied by the disadvantage that syringes of the metal to metal or metal to glass types have a great tendency to jam after the injection of a few millilitres of the mass. Several workers have overcome this difficulty by using apparatus of their own design, all basically similar and depending on compressed air or gas of some description for injection of the mass. Such is the apparatus of Narat, Loef & Narat (1936), Tobin (1947), and Trueta, Barclay, Daniel, Franklin & Prichard (1947).

Such technique is undeniably superior to the use of the syringe, even of the glass to rubber or fibre type, for large specimens giving, as it does, a comparatively even pressure over a considerable period. Nevertheless, some slight pressure fluctuation seems in our experience to occur when using this apparatus. We have succeeded in eliminating such fluctuation, providing at the same time a maximum pressure safety valve by inserting into the usual circuit, in lieu of the Winchester bottle or other reservoir, a simple device as follows (Text-fig 1).

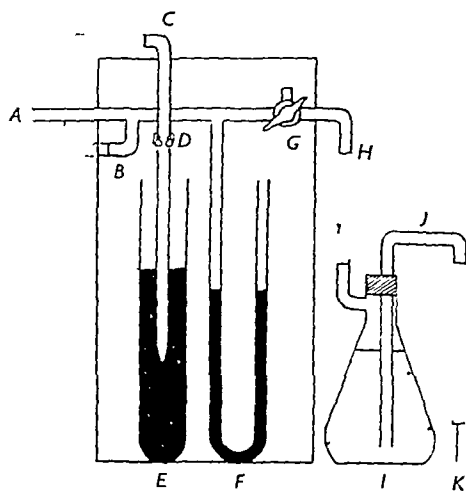


Fig 1 Rubber tubing connexions in broken outline

Compressed air enters at *A* and fills the apparatus. A T-piece *B* gives access via a length of rubber tubing to a length of glass tubing *C*. This tube *C* is held by a clip *D* through which it can slide up and down, and it dips into a large tube *E* containing mercury. According to the depth of immersion of tube *C* into the mercury so the compressed air has greater or less difficulty in escaping through the mercury, and

the pressure in the apparatus is registered by manometer *F*. At *G* is a three-way stopcock which allows the compressed air to flow via tube *H* to injection mass container *I*, or shuts off tube *H* from the rest of the apparatus, or allows the pressure of gas in tube *H* and container *I* to escape into the atmosphere, thus ensuring an immediate cessation of the flow of the injection mass from *I* through *J* to needle or cannula *K* whenever desired.

This apparatus has been in use for almost two years and has proved eminently satisfactory for the injection of corrosion specimens, the injection of indian ink and various radio-opaque substances into the kidneys of anaesthetized animals in physiological experiments, and for sundry other purposes requiring a known injection pressure in conjunction with some safety device to ensure against distortion of vessels from their normal paths by sudden raising of the pressure.

It is strongly recommended that as much as possible of the conducting part of the apparatus be made in one piece of glass as this eliminates several likely sources of air leakage, such as from rubber tubing joints on T-pieces, etc. For the same reason we prefer to use a Buckner funnel as a container in such injections as require only a litre or less of volume.

SUMMARY

The replacement in the compressed air type injection apparatus of the usual pressure stabilizing bottle by an open mercury reservoir into which dips a tube connected with the circuit conducting the main flow of compressed air combines, it is claimed, a constant pressure with an automatic maximum pressure safety valve.

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THE USE OF SAFRANIN-O FOR THE DEMONSTRATION OF CYTOPLASMIC NUCLEOPROTEINS

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The experiments to be described were originally designed to discover whether histochemical evidence for the presence of proteolytic enzymes or their precursors could be obtained. For this reason, attention was initially concentrated upon the zymogen granules of the pancreas and the pepsinogen granules of the peptic cells of the stomach. The animals used were rats and guinea-pigs.

The technique employed was based upon the fact that safranin and certain other related azine dyestuffs combine, apparently specifically, *in vitro*, with proteolytic enzymes. The first observations were made by Robertson (1907), who showed that when a drop of saturated Grubler's safranin was added to a solution of trypsin, a precipitate was formed. The observations were further extended by Holzberg (1913), who showed that the precipitate formed with trypsin possessed proteolytic activity. Marston (1923) demonstrated that the precipitate formed with a crude pancreatic extract possessed proteolytic, but neither lipolytic nor diastatic activity, he was also able to prepare trypsin, erepsin, pepsin and papain azine precipitates with safranin and other azine bases, such as neutral violet and neutral red. Marston suggested that the enzyme became linked to the dye through the basic nitrogen of the azine ring.

On the evidence from the *in vitro* experiments just mentioned, it was hoped that the perfusion of freshly killed animals with a safranin solution might result in the dye being 'fixed' in cells or regions of cells containing proteolytic enzyme systems.

It will be seen from what follows, that the expected results were not obtained, but that the dye was found to be firmly held only in cells the cytoplasm of which is known to contain a high concentration of ribose-nucleoprotein.

MATERIALS AND METHODS

The fourteen guinea-pigs and eight rats used in these experiments were killed with chloroform or ether. The thoracic aorta was exposed and cannulated, the right auricle punctured and the vascular system perfused with normal saline at 37°C for 10 min. The saline was immediately followed by a 1% solution of filtered Grubler's safranin in normal saline at the same temperature. [In later experiments this safranin had to be replaced by one of another manufacture and this was found to be less reliable.] The perfusion with the dye was carried out more slowly and lasted up to 20 min. and, on an average, 50 ml. of the solution were used. After perfusion, pieces of stomach, pancreas, kidney and, in later experiments, liver and pituitary gland were removed. All these tissues were fixed in 10% formalin, but some pieces of stomach, pancreas and kidney were fixed in absolute alcohol or Bouin's fluid. The tissues were washed overnight in water, dehydrated through the alcohols, paraffin embedded and cut at 10 μ .

Some sections were examined without further treatment, others were lightly counterstained with light green, and the nuclei stained with a 1 % aqueous solution of methyl green. Since acidified alcohol removes basic dyes from tissues, the effect of a 1 % HCl solution in 70 % alcohol upon the retention of the dye in the cells was also investigated.

In addition, one rat was perfused with a 1 % solution of neutral red in saline and another with a 1 % solution of neutral violet in saline. Also, a rat and a guinea-pig were perfused with a 1 % solution of methyl green in saline and one guinea-pig with a 0.5 % solution of toluidin blue in saline. These last two dyes were used as controls, since they are both basic substances but are chemically dissimilar to the azine dyes. Finally, small pieces of stomach and pancreas from an unperfused guinea-pig were fixed in 10 % formalin, washed in water, soaked in 1 % safranin solution in saline overnight, dehydrated, embedded and cut. This was done to discover whether safranin was retained in any particular cells *after* fixation.

In some later experiments, when it appeared that the safranin retention in cells was associated with the presence of nucleoproteins rather than proteolytic enzymes, it was decided to investigate the possible 'fixation' of the dye by the Nissl substance of neurones. Difficulty, however, was encountered in getting satisfactory results after perfusion of the nervous system. In consequence, a different technique was used. Pieces of medulla were removed from guinea-pigs killed with ether and 15 μ sections were immediately cut on the freezing microtome. These sections were placed for 30 min. in warm 1 % safranin in normal saline, after which they were rinsed in water and fixed in 10 % formalin overnight. The sections, after washing in water, were stained with light green in clove oil and mounted in balsam. Where the general cytoplasmic staining was strong, the dye was removed by treating the sections for 5 min. with acid alcohol before counterstaining.

RESULTS

Of the total of twenty-two perfusions carried out, those on three rats and five guinea-pigs were unsatisfactory. This was due either to a failure of the perfusion before the safranin was passed through, or to an incomplete perfusion of the dye.

Before the results are described in detail, it must be emphasized that these experiments are not considered to be comparable with those in which a dilute solution of a relatively non-toxic dye is applied to tissues *intra-* or *supra-vitally*. In such cases the cells may be regarded as being in a practically normal functional state. In these experiments, however, where the animals were first killed by a lipid solvent anaesthetic and where, on an average, half an hour elapsed before the perfusion was complete, it seems almost certain that some post-mortem changes would have occurred within the cells, particularly in their membrane permeability. On the other hand, there was no evidence to suggest that the post-mortem changes were such as to result in any gross displacement or alteration of intracellular structures such as secretion granules or chromidial substance.

The effect of the method of fixation on the retention of the dye

Perfused pieces of stomach and pancreas from one rat were fixed in absolute alcohol and from one rat and one guinea-pig in Bouin's fluid. In neither case was

there any fixation of the dye, which was lost from the tissues during the fixation process itself. Both of these fixatives are protein precipitants and therefore act in a different manner from formalin, after the use of which the dye was retained in certain cells to be described below.

The retention of safranin in the cytoplasm of certain cells after perfusion

Immediately after a satisfactory perfusion the whole tissue was a brilliant reddish purple colour, but during the course of fixation, washing and dehydration the dye largely diffused out. After cutting and the removal of paraffin from the sections, there was generally some diffuse staining of the section as a whole in addition to the more intense staining of those cells which appeared to have an unusual affinity for the dye. The diffuse staining generally disappeared during downgrading through the alcohols, prior to staining the nuclei with methyl green. Alternatively, the diffuse stain could readily be removed by treatment with acid alcohol for a minute or two, but such treatment did not affect the dye in the specifically stained cells.

The retention of safranin in gastric and pancreatic cells

The results obtained were rather unexpected, both in rats and guinea-pigs. It was anticipated that if there was to be any fixation of the dye in these particular organs its attachment would most likely be to the proteolytic enzyme precursors, i.e. the pepsinogen granules of the stomach and to the zymogen granules of the pancreas. In no instances, however, were these granules coloured by the safranin, although certain regions of the cytoplasm of both peptic cells and the alveolar cells of the pancreas were seen to retain the dye. The retention of the dye in these cytoplasmic regions was, moreover, quite strong, since in well-perfused specimens treatment for 12 hr. in 1% HCl in 70% alcohol did not completely remove the safranin from them, although downgrading through the alcohols, or a brief rinse in acid alcohol was sufficient to remove the dye from the rest of the section. One exception to this last statement was observed in the stomach, where the cytoplasmic granules of certain, as yet unidentified, connective tissue cells showed a strong affinity for the dye.

The distribution and morphology of the safraninophil material in the cytoplasm of the alveolar cells of the pancreas was particularly characteristic. It recalled the basophil material known variously as the chromidial substance or ergastoplasm. This material, as is well known, occurs in the form of filaments and granules in the proximal part of the cytoplasm, where the mitochondria are primarily concentrated. The correspondence (Pl 1, figs 3, 4, Pl 2, fig 10) between the chromidial substance as revealed by basic dyes in routine preparations *after* normal fixation, and the strands and granules coloured by the safranin when applied *before* fixation was so close, that there seemed little doubt that the structures so stained were identical. The secretion granules were uncoloured.

Again, in the peptic cells, the safranin-stained material was in the form of threads and granules (Pl 1, fig 1, Pl 2, figs 6-9) strongly reminiscent of the ergastoplasm. It was possible to stain the cells by Heidenhain's iron-haematoxylin method—a procedure which demonstrated the pepsinogen granules in the distal part of the cell and temporarily obscured the 'safraninophil' material in other parts of the

cytoplasm, on destaining, however, with iron alum, the red ergastoplasmic material reappeared. Thus once more, there seemed little doubt that the cytoplasmic parts stained corresponded closely with the basophil material known to occur in peptic cells

Recent studies using the ultra-violet light absorption technique (Caspersson, 1940), and the method of digestion with solutions of purified ribonuclease (Brachet, 1941) have produced powerful circumstantial evidence for the presence of a high concentration of ribose-nucleoproteins in these basophil ergastoplasmic regions of pancreatic and peptic cells (see Caspersson, 1947, pl 2, fig 2) and, indeed, for a large number of other cells known to have a high rate of protein synthesis (Caspersson, 1947, Thorell, 1947) In view of these findings, it was decided to investigate the fixation of safranin in certain other organs, the cells of which are known to possess cytoplasmic ribose-nucleoproteins Those chosen were liver, pituitary and brain, and the results obtained are described below

The retention of safranin in the liver, pituitary and brain

The highest concentration of safranin in the liver was in the cells at the periphery of the lobules close to the portal canals The safraninophil material was either in the form of aggregates of small deeply staining granules distributed at random in the cytoplasm or the whole of the cytoplasm was stained, but to a lesser degree (Pl 2, fig 5) The former appearance resembled most closely the basophil material described in liver cells

Although only one satisfactory perfusion of the guinea-pig anterior pituitary was obtained, the results are reported here In sections of this specimen it was not possible to distinguish the cell types with certainty, but strands and granules of safranin-staining material were observed in what appeared to be basophil and acidophil cells This was similar to the basophil material demonstrable in these cells after fixation in Helly's fluid, and staining with methylene blue-eosin mixtures

As has been pointed out above, it was found necessary to alter the technique for the study of the neurones of the central nervous system The essential condition of the other experiments was, however, maintained in that the tissue was subjected to the dye *before* fixation Examination of the sections showed unquestionably, that the safranin had become 'fixed' to the Nissl substance (Pl 1, fig 2), and this fixation was resistant to several hours' treatment with acid alcohol Furthermore, an examination of Auerbach's plexus in the safranin perfused stomach showed neurones with their Nissl substance dyed by the safranin

The retention of safranin in the kidney

The kidney was initially studied together with the stomach and pancreas, in order to see whether the use of safranin might be a method for the histological demonstration of the proteolytic enzyme renin Examination of the sections showed that the dye was held in certain tubules of the cortex, the medulla being very much less deeply stained After treatment with acid alcohol, the dye was still found to be held in the cells of the proximal, and possibly in those of the distal convoluted tubules, the staining reaction was diffuse and the dye did not appear to be attached to any threads or particles, as was the case with peptic and pancreatic cells In some

instances, the dye appeared to be adsorbed to the capillary walls of the glomeruli and to material precipitated in the cavity of Bowman's capsule

The effect of staining with safranin after fixation

The technique used has already been described. It was found that the dye was very rapidly extracted during the embedding process from the pieces of guinea-pig's stomach. The examination of sections, prior to downgrading through the alcohols, showed a coloration of all cells and nuclei, as well as a strong staining, this time of the pepsinogen granules. Alcohol and acid alcohol were found to remove the safranin from the cells and pepsinogen granules with great rapidity—a marked contrast to what was observed in the previous experiments.

The retention of neutral violet and neutral red

The fundus of the rat's stomach was used to discover whether these two dyes, chemically related to safranin, were likewise fixed in the peptic cells. In both cases there was a retention of the dye in these cells. The rather pale colour of the neutral violet made it difficult to determine the exact nature of the cytoplasmic parts stained, but there was certainly no evidence that the pepsinogen granules took up the dye. The reaction with neutral red was diffuse, but there was no staining of pepsinogen granules.

The effect of perfusion with methyl green and toluidin blue prior to fixation

These substances were chosen as examples of powerful basic dyes, chemically dissimilar to the safranins. It was thought that if the retention of the basic dye, safranin, in certain cells was merely due to a combination with an acid component in the cytoplasm, then other basic dyes might act in a similar manner. This was not the case, however, with the two dyes used, they were rapidly washed out during embedding and downgrading through the alcohols and were quickly and completely removed by acid alcohol.

The retention of safranin by nuclei

The results here were variable. In a number of instances, and particularly in nerve cells, it was noted that the plasmosomes and more irregular granules, interpreted as karyosomes, held the safranin strongly. The dye could often be masked with a nuclear stain such as methyl green, but the red colour reappeared after the removal of the green dye with acid alcohol.

DISCUSSION

From the results described above, the following principal points emerged.

(1) The safranin was firmly held, even after acid alcohol treatment by the cytoplasm of certain cells, when the subsequent fixation of the specimen was in formalin. It was not held when subsequent fixation was in alcohol or Bouin's fluid. Of these three fixatives only formalin is a non-precipitant of proteins.

(2) The application of the dye *after* fixation in formalin did not result in its retention by any cells in the tissues studied. In fact, in the stomach there was

a qualitative difference in that the pepsinogen granules were temporarily stained, whereas in safranin perfusion *before* fixation they were never coloured

(3) It did not seem likely that the holding of the dye was directly related to its basic nature, since the two chemically dissimilar basic dyes used—toluidin blue and methyl green—were rapidly removed, both during the embedding process and afterwards

(4) The chemically related dyes, neutral violet and neutral red, were also found to be held in cells similar to those which react strongly with safranin. The staining, however, particularly with neutral red, was more diffuse and its precise intracellular distribution was difficult to determine

Recent investigations by Schultz & Caspersson (1940), Caspersson (1947), Thorell (1947), and others, have provided evidence which suggests that the nucleoproteins of the cytoplasm play a very important part in intracellular protein synthesis. A high concentration of ribose-nucleoproteins appears to be associated, for example, with embryonic cells, developing blood cells and various gland cells possessing a high protein turnover. Moreover, it seems more than probable, that in various glandular cells of an exocrine type, the nucleoproteins are largely concentrated in the strongly basophil chromidial substance or ergastoplasm. This certainly seems to be true for pancreatic and peptic cells. The basophil material present in the chromophil cells of the pituitary and in liver cells, as well as the Nissl substance of nerve cells (Hyden, 1947), also contains a high proportion of ribose-nucleoproteins. In all these instances it is possible to destroy the characteristic basophilia by the incubation of tissue sections with the enzyme ribonuclease for which ribose-nucleoprotein is believed to be a specific substrate (Brachet, 1941, 1947, Dempsey & Wislocki, 1945, Davidson & Waymouth, 1943, Gersh & Bodian, 1943)

The close correspondence between the morphology and position of the cytoplasmic structures 'fixing' safranin in these experiments and the ergastoplasm and Nissl substance suggests that it is the nucleoproteins of the cell which are binding the dye. It is not necessarily true, however, that the dye combines specifically with ribose-nucleoproteins, since, as has been mentioned, the karyosomes (which contain desoxyribose-nucleoprotein) often showed a strong affinity for the dye. In nerve cells, the plasmosomes which contain ribose-nucleoprotein were always strongly reacting

It may be relevant that Dustin (1947) has suggested that certain vital dyes, such as neutral red, which accumulate in pre-existing and newly formed intracellular vacuoles (such as those of the Golgi zone of various cells), do so because they become attached to the acidic nucleoprotein molecules which he believes accumulate in these structures. There appears to be no suggestion, however, of any specific chemical attachment. Of more significance is the observation communicated to me by Prof Frank Dickens, F R S, to the effect that nucleic acids combine, *in vitro*, with phenosafranin (the substance of which safranin-O is a derivative) to form a precipitate

In an attempt to account for the observations which have been made, the two following alternative hypotheses are put forward. First, the nucleic acid part of the nucleoprotein molecule may combine with the safranin to give a fairly stable compound, which is not destroyed by formalin and which is resistant to subsequent treatment with acid alcohol. Secondly, if, as has been recently suggested by

Brachet (1947), the nucleic acids of the cytoplasm are associated with proteolytic and other enzyme systems, then the safranin may combine with these associated proteolytic enzymes, presumably by the same mechanism as operates *in vitro* (see Introduction) On this hypothesis, the failure of the dye to combine with pepsinogen or zymogen granules must be assumed to be due to the fact that these bodies contain enzyme precursors and not the active enzymes themselves, and in consequence behave differently

The results with kidney tissue are anomalous according to either hypothesis, since there is little ribose-nucleoprotein in kidney cells These cells are, however, unique in respect of their absorbing and secretory properties, and the diffuse retention of the dye by proximal convoluted tubules might be a special case connected with these properties, but since such an explanation is dependent upon the assumption that the kidney cells in these experiments were sufficiently undamaged to function normally, it seems an unlikely one

SUMMARY

1 Safranin and certain other azine dyes were found to be firmly held in the acinar cells of the pancreas, peptic cells of the stomach, liver cells, nerve cells of Auerbach's plexus and the chromophil cells of the anterior pituitary, following post-mortem perfusion with the dyes, and subsequent fixation of the tissues in formalin The dye was also retained in nerve cells of the brain after the special procedure already described

2 The intracellular distribution of the dyes and the distribution of the cytoplasmic nucleoproteins were found to be in close correspondence

3 It is suggested that the dye combines directly, either with the nucleic acids, or else with proteolytic enzymes which may be associated with them

I would like to thank Prof F Goldby of the Anatomy Department, St Mary's Hospital Medical School, for his interest and helpful criticism

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EXPLANATION OF PLATES

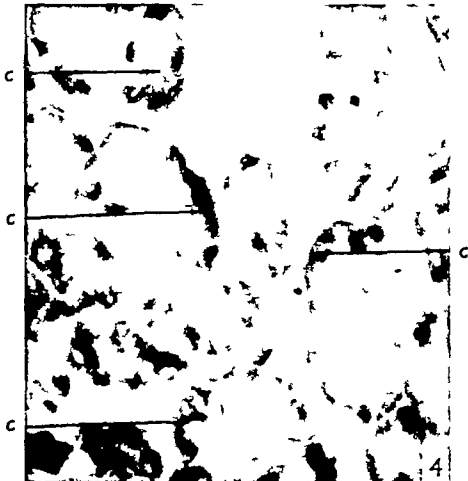
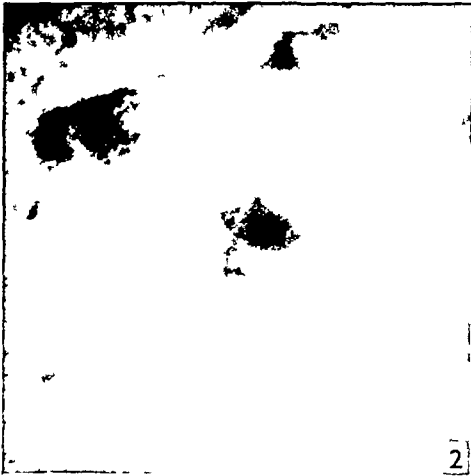
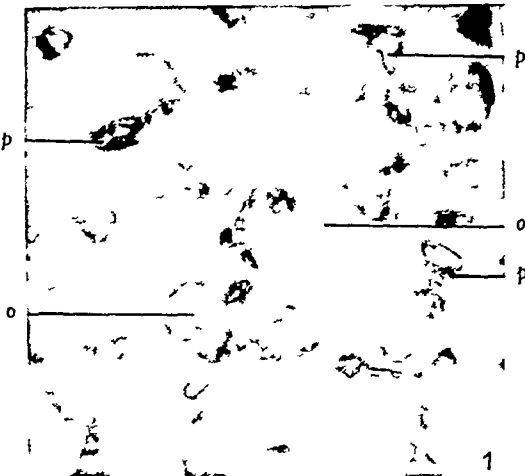
PLATE 1

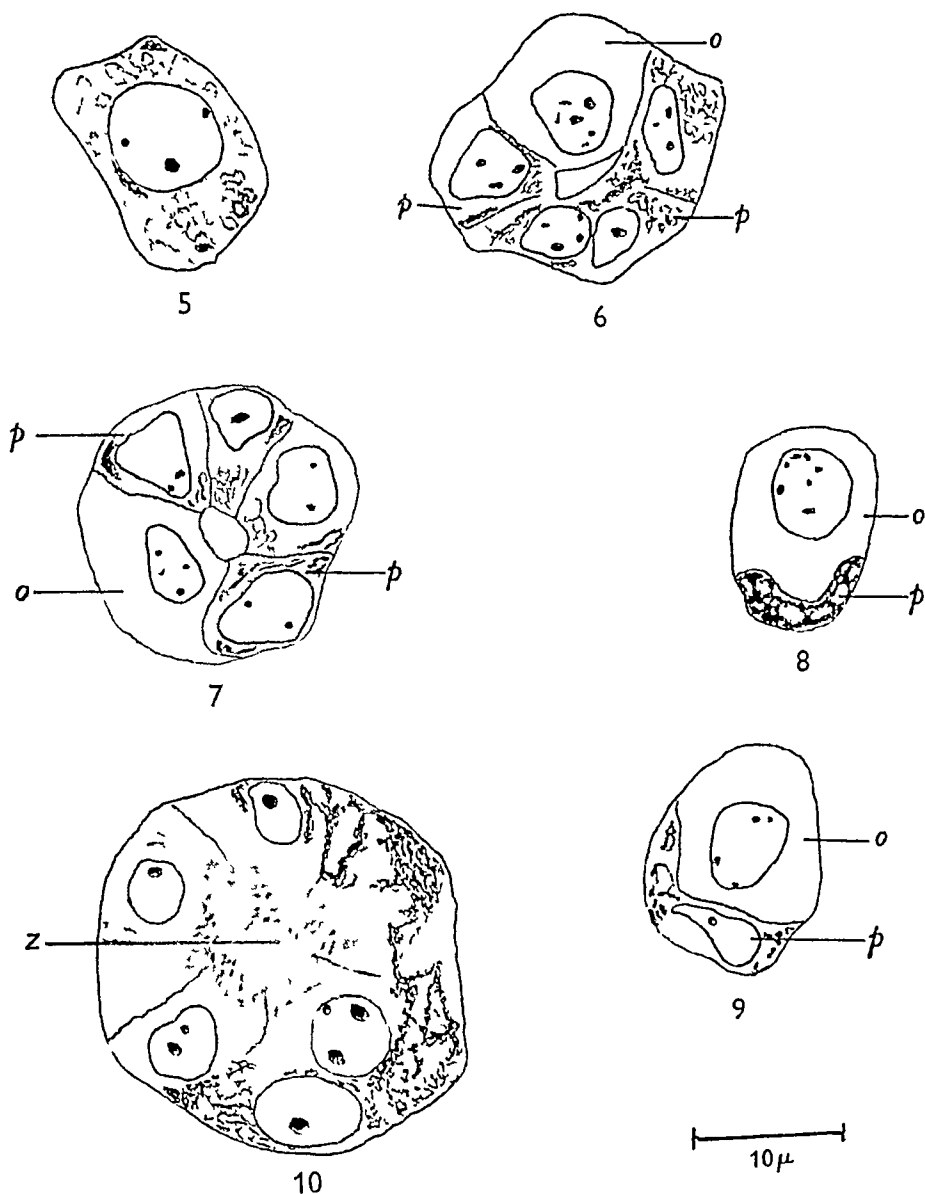
- Fig 1 Guinea pig stomach Peptic cells (*p*), oxyntic cells (*o*) Safranin perfusion, nuclei stained with methyl green $\times 1000$
- Fig 2 Nerve cells from medulla of guinea pig, showing retention of safranin in the Nissl substance Stained with safranin prior to fixation (see text), and counterstained with light green $\times 500$
- Fig 3 Pancreas of dog, showing distribution of chromidial substance (*c*), for comparison with fig 4 Zenker formol fixation, Ehrlich's haematoxylin and eosin $\times 400$
- Fig 4 Guinea pig pancreas, showing retention of safranin in the basal regions of the cells, where chromidial substance is normally present (*c*) Safranin perfusion, nuclei stained with methyl green $\times 600$

PLATE 2

Figs 5-10 Camera-lucida drawings of safranin perfused guinea pig material

- Fig 5 Liver cell showing distribution of safraninophil material
- Figs 6-9 Cells from fundus of stomach showing the distribution of the safraninophil material which is in the form of threads and granules in the peptic cells (*p*) Note that this material is absent from the oxyntic cells (*o*)
- Fig 10 Section of pancreatic alveolus Notice that the distribution of the safraninophil material corresponds with that of the chromidial substance (cf Pl 1, fig 4) *z*=region of unstained zymogen granules





DENDRITIC CELLS IN PIGMENTED HUMAN SKIN

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INTRODUCTION

It has long been known that the characteristic coloration of the skin of the pigmented human races is due to the presence of the pigment melanin. This occurs in the epidermis where it is most abundant at the level of the deepest or basal layer of cells. As to the source of origin of this pigment and the intimately connected problem of the exact cellular composition of the tissue that contains it, complete unanimity of opinion has not yet been reached, some authors still maintaining that the Malpighian cells are the site of melanogenesis (see reviews by Hoepke, 1927, Bloch, 1929, Percival & Stewart, 1930, Laterjet, 1938, Meirowsky, 1940, Masson, 1948).

In guinea-pig epidermis, a compound tissue in whose structure two anatomically distinct types of cell participate—Malpighian cells with their specialized derivatives, and dendritic cells—melanogenesis has recently been shown to be an exclusive property of the latter (Billingham, 1948). Two distinct 'true breeding' races of these branched cells exist: the 'pigmented' dendritic cells which occur in the black epidermis of the spotted black-and-white guinea-pig and which are responsible for its pigment formation, and the anatomically identical 'white' dendritic cells which are found exclusively in the white epidermal regions. 'Pigmented' and 'white' dendritic cells differ only in that the former are endowed with the power of melanogenesis. The epidermis of ordinary white human skin has a similar twofold composition, but so far as pigmentary activity is concerned it resembles the skin of the so-called albino guinea-pig in that it has a latent capacity for pigment formation. In this animal the epidermis is normally white, probably because of the presence of an inhibitor (Onslow, 1915, Ginsburg, 1944), but it may be caused to blacken by such a mild stimulus as cold weather. It may be added that in the white skin of the spotted guinea-pig no merely physical stimulus is known which will initiate even the slightest degree of melanogenesis (Lewin & Peck, 1941, Ginsburg, 1944).

In view of these considerations there seemed to be the strongest indirect evidence that the epidermis of pigmented human skin would have a functional and anatomical dendritic cell system closely resembling that present in pigmented guinea-pig skin. The object of this study has been to obtain direct evidence in support of this analogy.

In this paper the term 'pigmented human skin' refers only to that of Indian or Negro and no attempt has been made to differentiate between the two types. The two main techniques used, although briefly described in a previous paper (Billingham, 1948), are here reported in some detail, in the hope that they will be of use to other students of cutaneous pigmentation.

MATERIAL AND METHODS

Pieces of normal pigmented skin (made available through the kindness of Prof. T. Pomfret Kilner, Dr H. M. Hanschell and Mr Dallas Ross) from various sites on the bodies of Indians and Negroes have been used in this study. The bulk of the material

consisted of trimmings from Thiersch grafts (i.e. very thin sheets of skin comprising the epidermis and only the superficial part of the dermis)

Sections The fresh material was fixed in formol-mercuric chloride, dehydrated in an ethyl alcohol series and, after the use of cedarwood oil followed by ligroin as antemedium, the tissue was embedded in paraffin wax. Sections were cut at 8–10 μ and were lightly stained with Mayer's carmalum or Ehrlich's haematoxylin and eosin.

Whole mounts of 'Split' skin The skin-splitting technique adopted (Medawar, 1941) depends upon the enzymic dissolution of the fine elastic fibres that unite the epidermis to the dermis. Freshly obtained Thiersch graft shavings which had previously been vaselined on their cuticular surface were cut into fragments of approximately 0.5 cm². These were floated on to a Seitz-filtered 0.5% solution of commercial trypsin powder in Ringer-bicarbonate containing 1:100,000 phenol red and adjusted thereby to pH 7.8, and digested for about 30 min, or longer if necessary (depending on the thickness of the material), at 38° C. The fragments were then rinsed in unbuffered Ringer's solution, blotted free from excess fluid, and then carefully flattened out on a dry slide, cuticular surface lowermost. The thin layer of dermal tissue was then lifted off with fine forceps leaving the epidermis behind as a thin intact sheet. The sheets of 'pure' epidermis so obtained (see Pl. 1, figs 3–5) and referred to hereafter as 'split' skin were either fixed directly in formal-calcium (Baker, 1944), dehydrated in an ethyl alcohol series, cleared in clove oil and mounted in balsam, or immediately after fixation were treated with the 'Dopa' reagent.

The 'Dopa' reaction (See Bloch, 1929.) So far as the epidermis is concerned the Dopa reaction affords a perfectly valid method of selectively revealing those cells which possess an active melanogenic system or enzyme complex (Russell, 1939, Ginsburg, 1944). It is based upon the fact that when fresh or freshly formal-fixed epidermal tissue is placed in a suitably buffered solution of *l*-3,4-dihydroxyphenylalanine (Dopa), a likely precursor of melanin (Raper, 1927), certain cells are able to bring about its intracellular oxidation to Dopa-melanin, becoming intensely blackened in the process, i.e. they are Dopa-positive.

Sheets of pure epidermis immediately after splitting were fixed for about half an hour in formal-calcium, rinsed in distilled water and transferred to the Dopa substrate freshly prepared by adding 2 ml of Sorensen M/15 potassium dihydrogen phosphate solution (KH₂PO₄) and 8 ml of Sorensen M/15 disodium phosphate solution (Na₂HPO₄) to 25 ml of the stock 1:1000 Dopa solution in distilled water. The tissue, placed in an open vessel to allow free access of air, was incubated in this substrate at 38° C for half an hour, after which the substrate was replaced by a freshly made-up solution and the incubation was continued for a further 2½–3 hr. The reaction was at an end when the solution had taken on a sepia-brown coloration. After rinsing in distilled water the standard fixation and mounting procedure was carried out.

OBSERVATIONS AND CONCLUSIONS

Study of transverse sections of full thickness pigmented skin, irrespective of the site on the body from which it was derived, shows that although melanin granules occur at all levels throughout the epidermis, and in small quantities in the superficial dermis, it is always at about the level of the deepest Malpighian cells that the pigment

Tabular summary of the epidermal glial system in man and guinea-pig

Type of cell	Occurrence	Normal 'colorimetric' description	Pigmentary activity	Notes
Pigmentary dendritic cell	Coloured human skin, e.g. Indian or Negro	Pigmented or black dendritic cell	Has a high level of melanogenesis which is intrinsically maintained	Cannot be distinguished in unstained or un-Dopa'd preparations because melanin granules are golden brown in colour and relatively large. Dopa reaction is essential.
	Black guinea pig skin	Ditto	Ditto	Easily visible in unstained 'split' skin preparations (Billingham 1948) since melanin granules are black and very minute in size.
	White human skin	'White' or non-pigmented dendritic cell	Normally very slight or absent	Possesses necessary enzyme system for pigment formation but an inhibitor is present (Rothman <i>et al</i> 1946). Suitable stimuli e.g. ultra violet light, will initiate limited degree of melanogenesis (e.g. sun tan) probably because of destruction or removal of inhibitor.
	White skin of albino guinea pig	Ditto	Can be evoked by appropriate stimuli	Can be caused to blacken, e.g. after exposure to cold. Dendritic cells can then be seen in 'split' skin preparations (Billingham & Medawar, 1948). Possess necessary enzyme system for melanogenesis but an inhibitor normally present (Onslow, 1915; Ginsburg 1944).
Non-pigmentary dendritic cell	White skin of spotted black and white guinea pig? wherever there is 'recessive' spotting in rodents	'White' or non-pigmented dendritic cell	Absent and cannot be evoked	No form of physical stimulus known which will initiate any trace of pigmentation (Lewin & Peck 1941). Do not possess necessary enzyme system for pigment formation.

Synonyms: melanophore, melanoblast, chromatophore, stellate cell, clear cell, cellule amboceptrice

is found at its greatest concentration (Pl 1, figs 1, 3, Pl 2, figs 9, 10). Here it may be so abundant as to obscure the boundaries of the cells (Pl 2, figs 9, 10). The pigment granules are spherical, uniform in size and of a deep golden brown colour in transmitted light, so differing markedly from those of black guinea-pig epidermis, which are black. The capping and partial enveloping of the basal-layer cells by dense masses of these pigment granules is as prominent here (Pl 1, fig 3, Pl 2, fig 9) as in the guinea-pig. In such preparations absolutely no indication of the presence of dendritic cells can be found.

In sections of 'split' skin, in the preparation of which the tryptic digestion process had been prolonged to the point at which there was a tendency for individual epidermal cells to break away from the epidermal sheet, dendritic cell processes can often be seen still applied to it and apparently anchoring isolated basal-layer cells to the sheet (Pl 1, fig 6, Pl 2, figs 10, 11)

In unstained whole mounts of 'split' skin, examined with their cuticular surfaces lowermost, careful search rarely if ever reveals a trace of a dendritic cell (see Pl 1, fig 4) This is in contrast to similar preparations of guinea-pig skin in which the pigmented dendritic cells stand out boldly by virtue of their content of opaque melanin granules which also enable the processes arising from their perikarya to be followed out to their finest terminal twigs

The Dopa-treated 'split' skin preparations have been the mainstay of this study Examination of these reveals that dendritic cells are present in exactly the same position and abundance as are their 'white' homologues in white human skin from corresponding regions Their perikarya occur at about the same level as the basal-layer cells (Pl 1, figs 2, 7, 8) and, as a single layer of discretely scattered cells among the latter, they follow faithfully the complex 'hill and valley' relief pattern which the lower surface of the separated epidermal sheet presents (Pl 1, figs 4, 5, Pl 2, fig 12) In this pattern the 'hills' are the downward prolongations of the epidermis into the dermis while the 'valleys' are really the spaces which were formerly occupied by the upward projection of the dermal papillae which, especially in thigh skin, tend to have flattened tops above which the epidermis is at its minimum thickness The dendritic cells are found in highest concentration along the summits and sides of the downwardly directed epidermal ridges, while along the 'valleys' corresponding to the shallowest and thinnest regions of the epidermis they are more sparsely distributed In these latter regions the branching systems arising from the individual dendritic cells tend to ramify more in the horizontal plane, thus enabling their relationships to neighbouring Malpighian cells and to cells of their own type to be studied (Pl 2, figs 12, 14) These preparations also reveal that a proportional relationship exists between the degree of epidermal pigmentation and abundance of dendritic cells

The epidermis of pigmented human skin is thus identical in its anatomical constitution to nonpigmented epidermis (see Pl 2, fig 13), being composed of two types of cell Malpighian cells and dendritic cells Functionally, of course, the dendritic cells in pigmented skin differ from those in white skin in that they maintain in the epidermis a constant and relatively high concentration of pigment, while the non-pigmented dendritic cells in white skin can only effect a perceptible pigmentation of the epidermis as a result of some form of external stimulus, e.g. ultraviolet light, irritants, etc This pigmentation never reaches a very high level of concentration and for its indefinite duration is dependent upon the continued application of the external stimulus Rothman, Krysa & Smiljanic (1946) have suggested that white human epidermis contains a factor, probably sulphydryl compounds, with an inhibitory action on melanogenesis On the basis of this evidence they have suggested that in melanoblasts (dendritic cells) both substrate and active enzyme are present but no reaction takes place between them because of the presence of the inhibitor Melanogenic stimuli act by oxidizing or otherwise destroying the inhibitor, thus allowing

melanogenesis to take place. This would account for the similarity, with respect to pigmentary activity, between white human skin and that of the albino guinea-pig which has previously been referred to.

These pigmented dendritic cells in human material are very similar both anatomically and functionally to the dendritic cells found in the black skin of the guinea-pig. They differ from the latter in that they cannot normally be distinguished by virtue of their own melanin content, either in sections or in 'split' skin preparations, the use of the Dopa reaction, which is a sensitive colorimetric test for their melanogenic system, is essential. This difference is probably due in large part to the intense black colour and fine grain of guinea-pig melanin compared with the golden-brown colour of the human pigment granules.

As in the case of the pigmented skin of the guinea-pig there is ample evidence that melanogenesis is an exclusive property of these pigmented dendritic cells and that the pigment granules present within the ordinary basal-layer or Malpighian cells, often in considerable numbers, are derived at second hand from the dendritic cells by a process which may almost be compared to injection. As Masson (1948) has pointed out, these cells are both secretory and excretory in function and may therefore be considered as glandular cells. Unlike endocrine and exocrine glands, however, their product, melanin, is secreted into other cells across the terminal end-caps of the dendritic cell branches. Masson has called this 'cytoocrine' activity.

In mammalian epidermis dendritic cells comprise a definite cell system as definite and specific in its own right as the reticulo-endothelial system. Billingham & Medawar (1948) have called it the *epidermal glial system*. To avoid any confusion which may have arisen with respect to terminology, a comparative summary of the 'epidermal glial system' in man and the guinea-pig has been included (see p. 111).

SUMMARY

1 It is shown that the pigmented epidermis of Indian and Negro skin is exactly similar in its anatomy to that of the white races, being composed of the more or less rounded Malpighian cells with their derivatives and the branched dendritic cells.

2 There is, however, a difference in functional activity between dendritic cells in the two types of epidermis, in that those present in pigmented tissue, like those of the black guinea-pig, have a high rate of melanogenesis which is intrinsically maintained, while those in white epidermis are capable only of a relatively slight degree of pigmentary activity as a consequence of the application of suitable external stimuli.

3 Attention is drawn to the glandular or 'cytoocrine' role of pigmented dendritic cells.

The author wishes to express his sincere gratitude to Prof. P. B. Medawar who supervised the work recorded in this paper and gave great assistance at all stages, and to Miss Jean Morpeth who has given invaluable technical assistance.

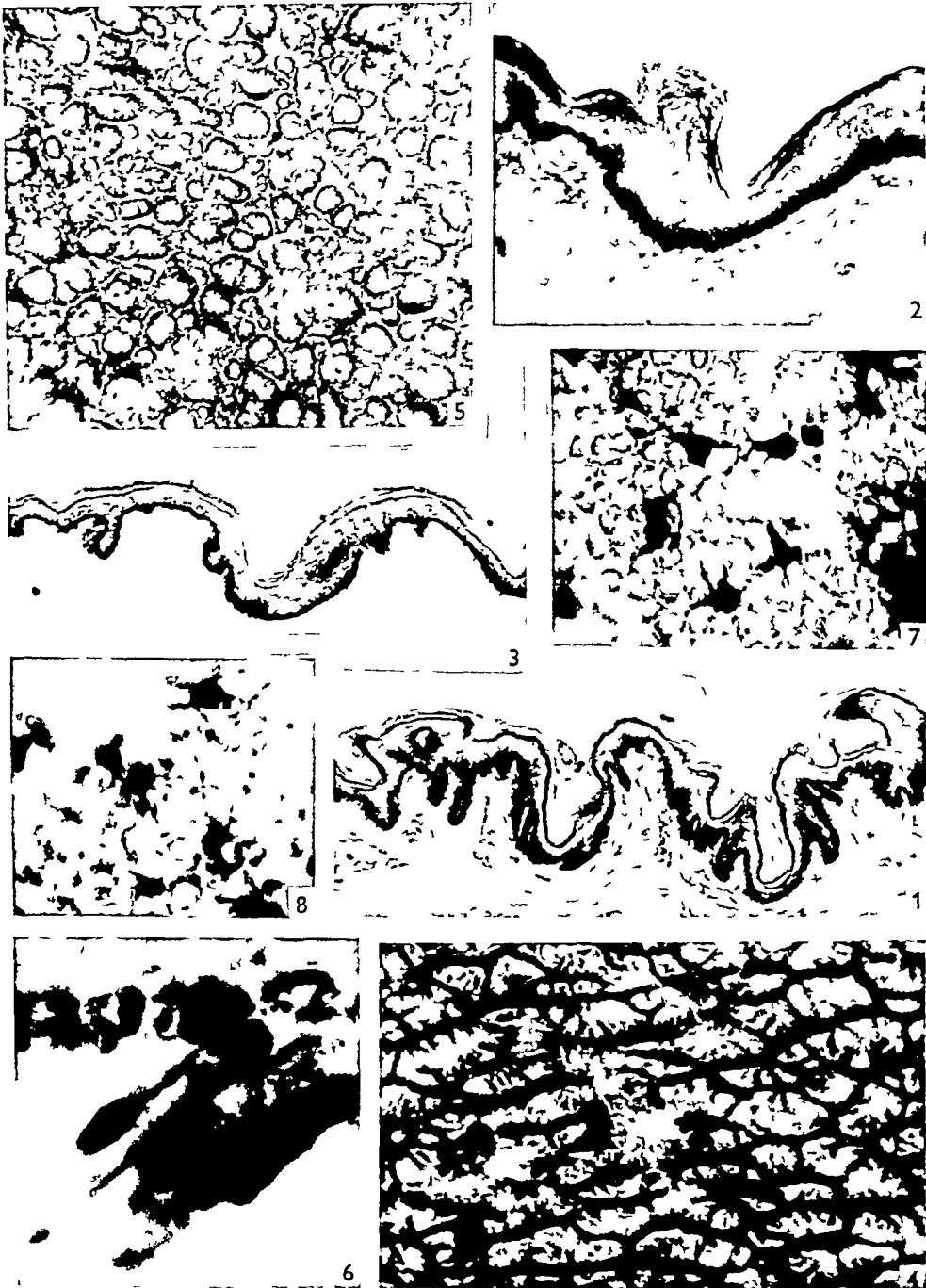
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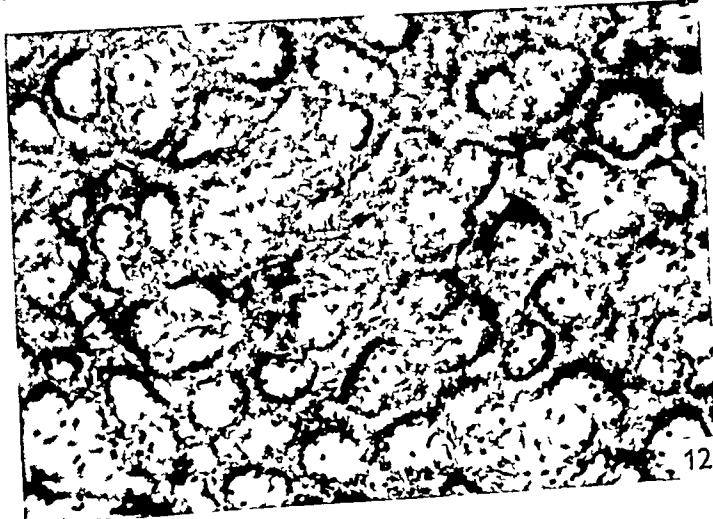
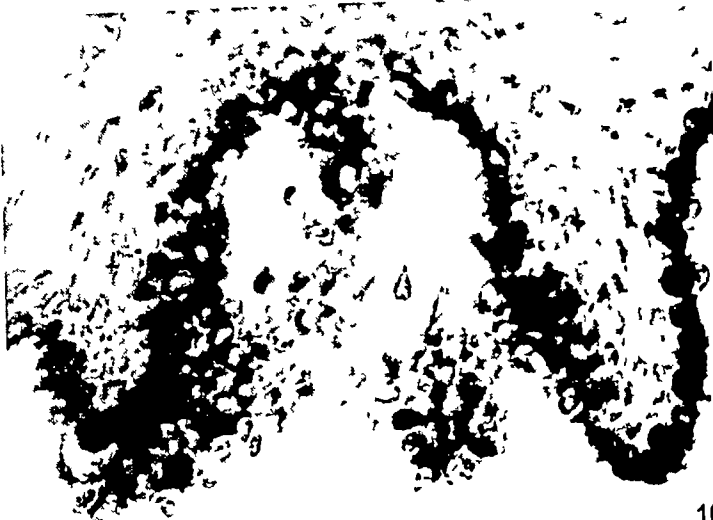
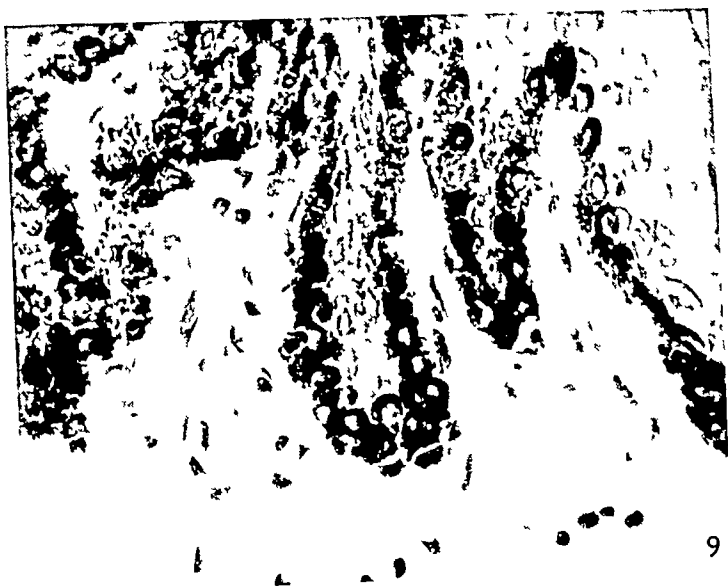
EXPLANATION OF PLATES

PLATE 1

- Fig 1 Vertical section through pigmented skin of Negro's scrotum Observe the complex corrugation pattern characteristic of scrotal skin The epidermal ridges penetrate rather deeply into the underlying dermis Pigment density is greatest at the level of the lowermost cells of the epidermis The dermis is almost devoid of melanin Lightly stained with Ehrlich's haematoxylin and eosin $\times 58$
- Fig 2 Vertical section of pigmented scrotal skin of Negro cut at 40μ on the freezing microtome and treated with Dopa The bodies of the dendritic cells and their processes can just be distinguished due to the intracellular formation of black Dopa melanin In the epidermis Dopa positive cells are only found at the level of the Malpighian layer $\times 75$
- Fig 3 Vertical section of 'split' pigmented scrotal epidermis The epidermis has been separated from the dermis by tryptic digestion and the sections prepared from it lightly stained with Ehrlich's haematoxylin and eosin (compare with fig 1) Although pigment is mainly restricted to the level of the basal layer cells, many of the epidermal cells at higher levels are 'capped' with pigment in a highly characteristic manner $\times 67$
- Fig 4 Whole mount preparation of a sheet of pure pigmented epidermis ('split' skin) of Negro's scrotum, unstained and viewed from the underside Note the complex 'hill and valley' contour or ridging pattern which the lower surface of the epidermis makes with the dermis This is often characteristic and specific for the different types of epidermal tissue found throughout the body (compare with that of thigh skin, figs 5 and 12) The 'hills' are the epidermal ridges while the 'valleys' are the spaces originally occupied by the dermal papillae In this preparation no indications of dendritic cells can be distinguished Pigment is mainly concentrated along the crests and sides of the epidermal ridges $\times 27$



BILLINGHAM—DENDRITIC CELLS IN PIGMENTED HUMAN SKIN



BILLINGHAM—DENDRITIC CELLS IN PIGMENTED HUMAN SKIN

- Fig 5 Whole mount of 'split' thigh skin of Indian which has been treated with Dopa. The preparation is viewed from the underside. Observe and compare the ridging pattern with that of scrotal epidermis (fig 4). The dendritic cell bodies have been 'stained' black by the Dopa and can be seen clearly. They are more closely distributed in the epidermal ridges which are also the regions of maximum depth of pigmentation. $\times 50$
- Fig 6 Vertical section of 'split' pigmented scrotal skin. One of the ordinary basal layer cells has almost broken away from the epidermal sheet, being retained only by a process from a dendritic cell which ends upon it as an intimately applied cap or end button. Unstained. $\times 1080$
- Figs 7, 8 Pigmented dendritic cells in a sheet of pure epidermis from Negro's scrotum which has been 'stained' with Dopa. Their perikarya and the processes which arise from them are intensely blackened by Dopa melanin. Note the melanin granules in the cytoplasm of the Malpighian cells in fig 8. $\times 383$

PLATE 2

- Fig 9 Vertical section of pigmented skin of negro's scrotum to show the highly characteristic 'capping' distribution of melanin in relation to the basal layer cells of the epidermis. Lightly stained with Ehrlich's haematoxylin. $\times 383$
- Figs 10, 11 Vertical sections of 'split' pigmented scrotal skin lightly stained with Ehrlich's haematoxylin and eosin. The enzymic splitting process was deliberately prolonged to the point at which cells had begun to separate from the epidermal sheet. Dendritic cell processes can be seen still adherent to some of the Malpighian cells which are breaking away. Note also the almost complete localization of pigmentation to the level of the basal layer cells of the epidermis. Fig 10, $\times 383$, fig 11, $\times 383$
- Figs 12, 14 Pigmented dendritic cells in pure pigmented epidermis of Indian's thigh skin stained by the Dopa method. At the bottoms of the epidermal 'valleys' the dendritic cells though more sparsely distributed tend to have their branches spread out more in the horizontal plane. Fig 12 $\times 80$ fig 14, $\times 120$
- Fig 13 White dendritic cells of split white (Caucasian) human skin. The preparation has been stained supravitaly with methylene blue solution in Ringer. Note that the distribution, size, number and mode of branching of the dendritic cells is just the same as with pigmented skin (compare with figs 12 and 14). $\times 150$

OBSERVATIONS ON THE SNOOT OF *VARANUS*, AND A COMPARISON WITH THAT OF OTHER LIZARDS AND SNAKES

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INTRODUCTION

The family Varanidae comprises the single genus of monitor lizards which are widely distributed throughout the tropical and subtropical regions of the Old World. This genus includes the largest of all living lizards, the different species are conservatively modified in adaptation to terrestrial, partly arboreal and partly aquatic habits. *Varanus* occupies a somewhat isolated position among the living members of the order Sauria, it has generally been recognized as an ally of the extinct mosasaurs (Williston, 1925, Camp, 1923, 1940), and has also been regarded as related to the ancestors of snakes (Camp, 1923, Romer, 1945). In spite of the interest of the group, however, no systematic investigation of the chondrocranium of *Varanus* has yet been made, although several studies of the bones and other cranial structures are available. Kesteven (1940) has made some observations on the development of the skull in *V. varius*, but these are restricted to the base of the cranium. Pratt (1948) has described and figured the nasal and vomeronasal organs of *V. monitor* and *V. niloticus*, his account, however, is necessarily brief as it forms part of a general survey of the nasal region throughout the Sauria. It was therefore thought desirable to attempt a more detailed study of the anatomy of the snout in this genus, and to compare conditions present in *Varanus* with those in other lizards and snakes. Since no general description of the snout of any reptile is available, it was also hoped that this account might be of wider interest.

This investigation is principally based on serial sections through the nasal region of a juvenile example of each of the following species: *V. monitor* (Linn.), (*syn. V. bengalensis*), *V. salvator* (Laurenti) and *V. niloticus* (Linn.). I am much indebted to Mr C. W. M. Pratt for the loan of his sections of *V. niloticus*, and also for opportunities for discussing my results with him. The graphic reconstructions and most of the sections figured are based on the specimen of *V. monitor*, which was sectioned at 14 μ and stained with haematoxylin and eosin. This specimen measured approximately 240 mm. from snout to tail-tip (head-length 25 mm.) and was obtained near Meiktila in central Burma. Other *Varanus* material included the head of an adult specimen of *V. salvator*, a skull assigned to the same species, a juvenile skull of *V. monitor* and another of *V. niloticus*. I am indebted to Mr H. W. Parker and Dr Malcolm Smith of the British Museum of Natural History, and to Dr R. E. Rewell and the Zoological Society of London for the gift or loan of these latter specimens. My thanks are also due to Mr W. Warwick James for the young specimen of *Tupinambis teguixin* from which the sections shown in Text-fig. 11C and Pl. 1B were prepared.

TERMINOLOGY

In a monograph on the snout of Crossopterygian fishes and lower gnathostomes in general, Jarvik (1942) has pointed out the shortcomings of the nomenclature usually employed in describing the structures in this region. In particular, he draws attention to the fact that inadequate distinction is usually made between cavities and the structures, both skeletal and non-skeletal, which enclose them. Since Jarvik's terminology seems to be an advance on any previously used and may, with certain modifications, be applied to vertebrates in general, it has been employed as far as possible throughout this account.

The endocranial skeleton of the nasal region is termed the nasal capsule. Inside this lie the nasal sacs which can be subdivided into various regions, as described by Beecker (1903) and Pratt (1948). Each nasal sac communicates with the outside by means of an anterior nasal tube opening by an anterior external nostril and with the mouth or pharynx by an internal nasal or choanal tube. The nasal and oro-pharyngeal openings of each choanal tube are termed the inner and outer choanae respectively, following the usage of Born (1879), who gave the first comprehensive description of this region in Sauria. Both anterior and internal nasal tubes pass through fenestrae, first in the endocranial and then in the dermal skeleton, the fenestrae for the anterior nasal tubes are termed the fenestra endonarina anterior and fenestra exonarina anterior respectively, while those for the choanal tubes are termed fenestra endochoanalis and fenestra exochoanalis respectively. The posterior nasal tubes and posterior external nostrils of fishes are regarded by Jarvik as homologous with the lachrymal ducts and puncta of tetrapods. In the case of Squamata the term lachrymal duct is preferable to the more familiar naso-lachrymal of mammalian anatomy, since in the former group its anterior opening is more closely related to the mouth than to the nasal sac in many forms.

Since the variations in the saurian palate, which have been almost entirely neglected in the English literature, appear of considerable importance in a general understanding of palatal morphology in amniotes, the terminology of this region has also received some attention. An attempt has been made to apply the principles of Jarvik's nomenclature to the palate, which may be defined as all those tissues which lie between the nasal sacs above, and the mouth or pharynx below. These tissues may be subdivided into

(a) The floor of the nasal capsule, which is conveniently described by the usual terminology of the chondrocranium

(b) The dermal skeleton, here called the bony palate

(c) The soft tissues lying between the bony palate and the oral and pharyngeal cavities, the term superficial palate has been arbitrarily applied to these

The duct of Jacobson's organ passes through these palatal tissues in all adult Squamata, and opens into the oral cavity, its epithelium becoming continuous with that of the superficial palate. The fenestrae in the endocranial and dermal skeletons traversed by this duct have therefore been called the fenestra vomeronasalis interna and fenestra vomeronasalis externa respectively.

CONDITIONS IN *VARANUS*(1) *Bones*

The skull bones of *Varanus* have been thoroughly described by Bahl (1937) with special reference to the individual bony elements and nerve foramina, and by Mertens (1942) who devoted much attention to interspecific variations. The bony skull has not, therefore, been treated in detail in the present account, although some attention has been given to certain features which have previously received little notice. No attempt has been made to make graphic reconstructions of the bones, but these elements have been drawn in from a dried skull of another juvenile specimen, so that their relationship with the chondrocranium may be appreciated (Text-figs 1A, 2A and 3). The levels of the individual bones in these drawings have been checked as far as possible to correspond with those in the sections on which the reconstructions of the chondrocranium have been based, in some cases slight alterations in the outlines of these bones have been made. The specific features of the bony skull described have been included with the accounts of the non-bony structures related to them, while the palate has been dealt with in the comparative section.

(2) *Nasal capsule*

The general morphology of the saurian chondrocranium is now well known from the work of Born (1879), Gaupp (1900), Rice (1920), de Beer (1937) and others, it is, therefore, only necessary to describe in detail the features which are particular to this genus. The reconstructions given (Text-figs 1A, 2A and 3) agree in the main with that of Pratt. The nasal capsule is narrow and elongated, corresponding with the contours of the head. As in all living reptiles it remains unossified throughout life.

The unfenestrated nasal septum is low and terminates anteriorly in a flattened rostral process which is expanded into lateral wings. These wings are upturned, especially in *V. salvator* (Text-fig 1D) and support the medial ethmoidal nerves and vessels. In *V. monitor* the wings taper off behind into the central part of the rostral process, which widens out again further back as the lamina transversalis anterior. The medial ethmoidal nerve and terminal branch of the ethmoidal artery run

Legend to Text-fig 1

Text fig 1 A *Varanus monitor*, subadult. Reconstruction of chondrocranium (right), and bones of snout (left), seen from above. (Bones in Text figs 1A, 2A and 3 drawn from dried skull.) Levels of sections in Text figs 4, 5 and Pl 1D are shown. B *Xenopeltis unicolor*, subadult. Dorsal view of bones of front of snout (drawn from dried skull). Levels of some sections in Text fig 12 are shown. C *Varanus salvator*, subadult. Reconstruction of part of snout showing relationship of ethmoidal nerves and vessels to the skeleton. The main nerve branches only are shown. The right half of the premaxillary bar has been removed. In this species the medial ethmoidal nerve does not enter the premaxilla before dividing into its terminal branches, as in *V. monitor*, but runs forwards in a groove on the dorsal surface of the bone. A lateral branch passes downwards through a large foramen between the premaxilla and maxilla, while medial branches run through a foramen between the premaxillary bar and the lateral part of that bone. The foramina for the corresponding nerve branches are differently situated in *V. monitor*, and study of the figures shown by Mertens (1942) suggests that the precise relationships exhibited by the structures in this region are liable to considerable interspecific variation. D, E *V. salvator*. Transverse sections through rostral process and premaxillary bar at levels shown in C above. F *Tarentola mauritanica*, adult. Dorsal view of bones of front of snout, drawn from a dried skull. The nasal and part of the maxilla have been removed on one side to expose the septomaxilla and bony palate. Level of section in Text fig 11B is shown (A, $\times 13$, B, $\times 5$, C, $\times 12$, D, E, $\times 10$, F, $\times 3.7$).

forwards between the medial edge of the anterior cupola and the rostral process, so that there is no distinct foramen apicale (Text-figs 1A, 2A, 3 and 4A,B). In *V. salvator* and *V. niloticus* these relationships are slightly different. The wings join the medial edge of the anterior cupola behind, and the nerves and vessels pass through an elongated foramen apicale between the wings and central part of the rostral process (Text-fig 1C-E).

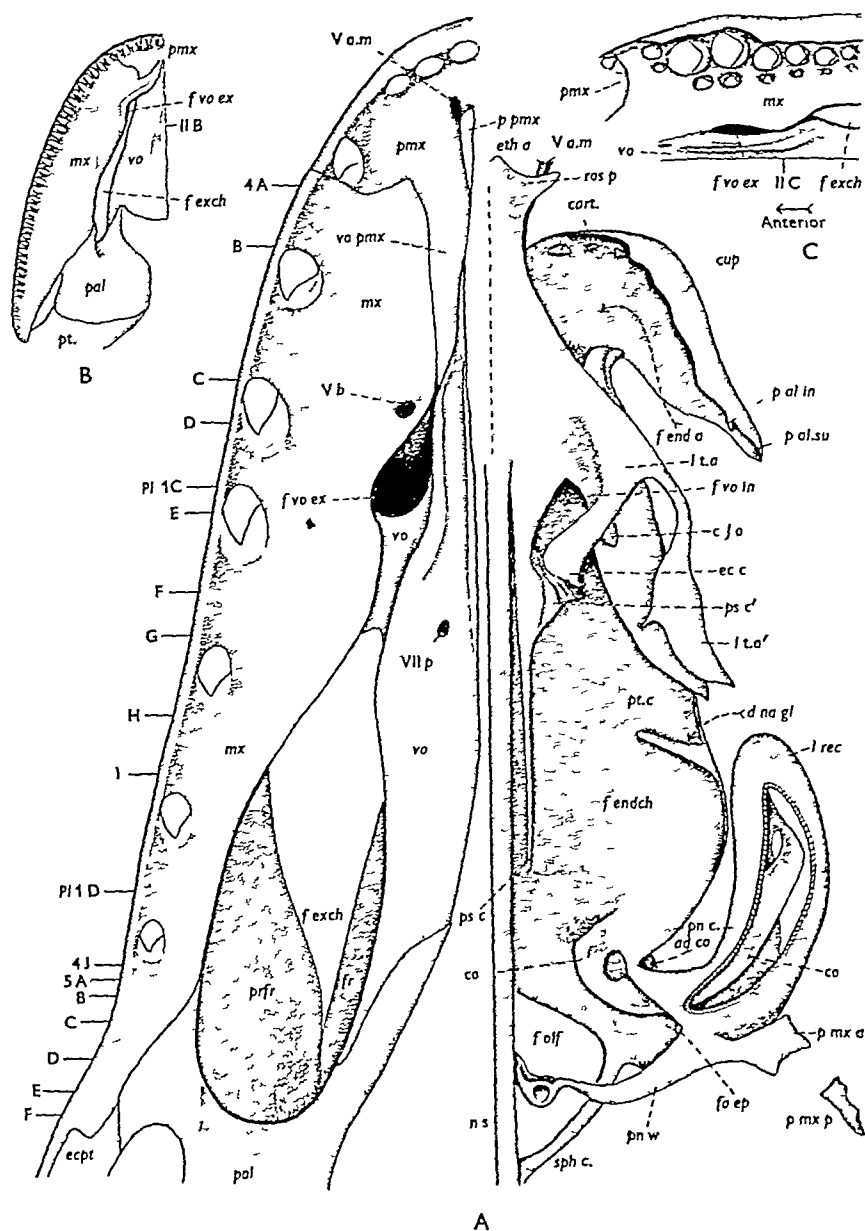
According to Pratt, the roof and sides of the nasal capsule are developed from two morphologically distinct elements—a medial element, the dorsal plate, which runs the whole length of the capsule roof on either side of the midline, and a lateral element which includes the parietotectal and paranasal cartilages which are continuous with each other. The line of junction between these medial and lateral elements cannot be determined accurately in the adult, but Pratt claims that it is indicated by the positions of the foramina for the branches of the ethmoidal nerve, and by the sites of the fenestrae superior and lateralis in those forms where they are present. In *Varanus* these fenestrae do not occur and it is only possible to infer approximately what the relative extent of these different elements may have been in the embryo. The labelling of the different regions of the capsule roof must therefore be regarded as provisional.

The front part of the capsule roof forms the large anterior cupola which lies above the curved part of the anterior nasal tube. The cupola is continuous laterally with the processus alaris superior, beneath which the anterior nasal tube passes inward to reach the nasal sac. A small projection from the cupola side wall beneath the processus alaris superior is interpreted as an inferior alar process (Text-figs 3, 4D). The fenestra endonarina anterior curves round above this small projection, behind and beneath the processus alaris superior (Text-figs 2A, 3).

Behind the anterior cupola the roof of the nasal capsule forms an uninterrupted covering for the elongated anterior chamber of the nasal sac. Owing to the characteristic incompleteness of the dermal skeleton of the varanid snout, this part of the capsule roof, together with the cupola, is exposed in the gap between the maxilla laterally and the narrow premaxillary bar in the midline (Text-figs 1A, 3 and 4A-H). The nasal capsule is therefore covered dorsally only by skin and connective tissue in this region.

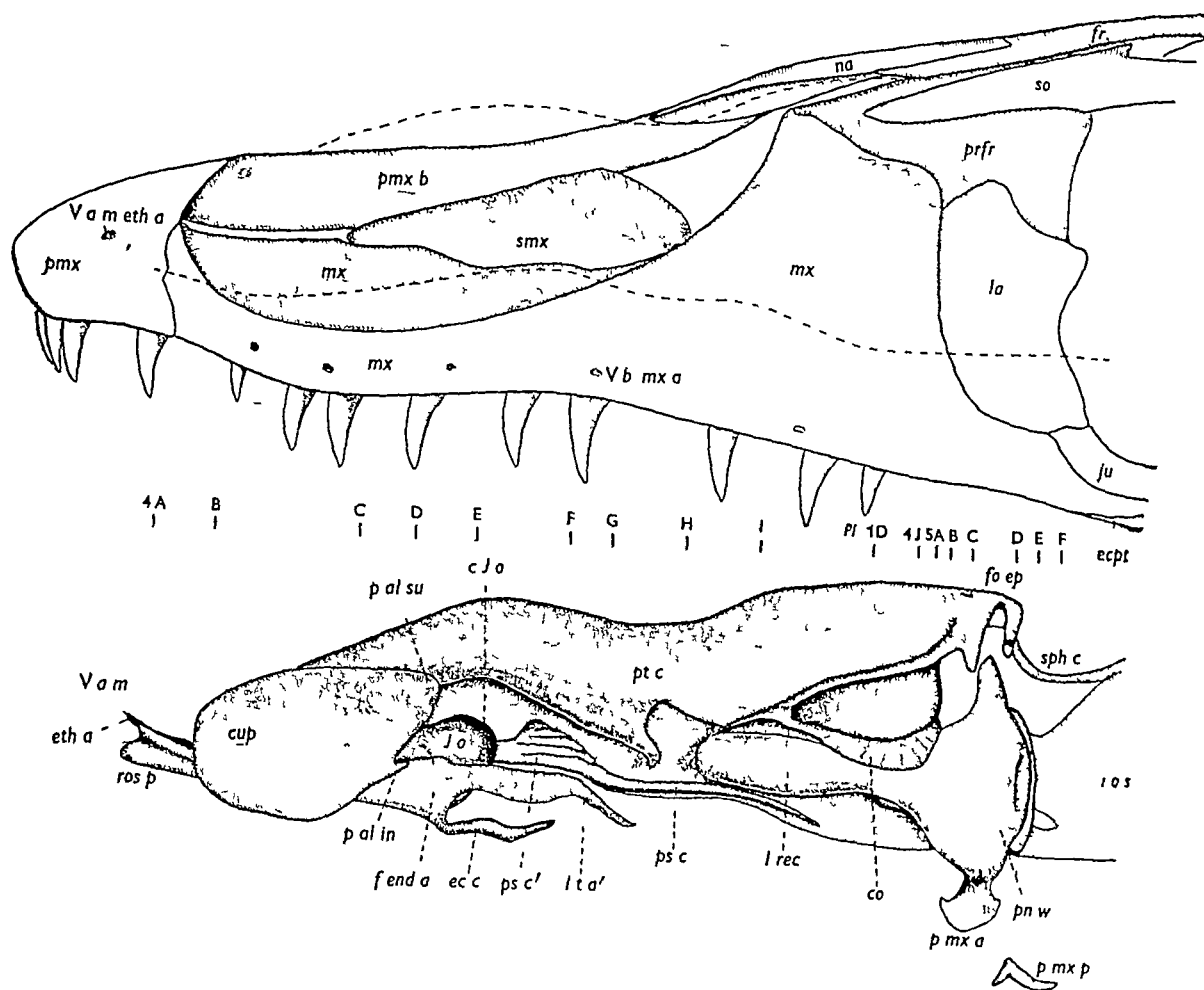
The middle part of the tectum nasi is expanded both laterally and dorsally to accommodate the olfactory chamber of the nasal sac (Text-figs 4I-J, 5A,B). Behind this level the roof is continuous laterally with the dorsal wall of the capsule of the lateral recess. The foramen epiphaniale is situated in the transverse segment which joins the main part of the roof of the capsule with that of the lateral recess (Text-figs 1A, 5C). The sphenethmoid commissures arise from the free posterior borders of the capsule roof (Text-figs 1A, 5D), the olfactory lobes project over the commissures into the fenestrae olfactoria.

Behind the region of the anterior cupola, the lateral wall of the nasal capsule only extends down as far as the upper part of the nasal septum. No zona annularis is present, and the cartilage of Jacobson's organ and the whole length of the parascptal cartilage are exposed in lateral view (Text-fig 3). Two processes project downwards from the free edge of the lateral wall. The first of these, situated about on level with the tip of the lamina transversalis anterior, marks the termination of the lateral part



Text fig 2 A *Varanus monitor*, subadult Reconstruction of chondrocranium (right) and bones of snout (left), seen from below Levels of sections in Text figs 4 5 and Pl 1C D are shown A window has been cut in the lateral recess capsule to expose the nasal concha B *Tarentola mauritanica*, adult Ventral view of right side of bony palate showing paleochoanate condition (Drawn from dried skull) Level of section in Text fig 11B is shown C *Tupinambis nigropunctata* adult Ventral view of right side of bony palate showing incomplete neochoanate condition (Drawn from dried skull) Level corresponding to section in Text fig 11C is shown (A $\times 13$ B, $\times 4$ C $\times 22$)

of the floor of the nasal capsule which runs to a point posteriorly (Text-fig 2A) The second process lies farther back at the level of the front of the lateral recess capsule The duct of the nasal gland (Text-figs 4I, 10C) hooks medially round this process before entering the nasal sac



Text fig 3 *Varanus monitor*, subadult Reconstruction of chondrocranium (below) and bones of snout (above), seen from the side Outline of roof of nasal capsule and lower edge of nasal septum is shown by interrupted lines in upper drawing Levels of section in Text figs 4, 5 and Pl 1D are shown The prefronto maxillary contact in the specimen drawn is unusual, in most specimens of *V monitor* the contact runs obliquely downwards from in front, and the maxilla sends up a spur which articulates also with the frontal and tip of the supraorbital ($\times 12$)

The posterior third of the lateral wall is modified in relation to the conchal infolding and the formation of the capsule of the lateral recess * The morphology of this region was found to differ considerably in the three species of *Varanus* examined

*This structure, which is present in many other lizards (see later) has usually been termed the *cavum extra conchale* or *extra conchal recess* Since the nasal concha extends into it in *V monitor*, however, the term *lateral recess* has been thought more appropriate

In *V. salvator* the lateral recess capsule is joined with the lateral wall of the main nasal capsule, and the concha is not continued round into the recess (Text-fig 10A). In *V. niloticus* conditions appear to be intermediate between those in *V. salvator* and *V. monitor*, the lateral recess is more extensive than in the former species and its anterior part is cut off from the rest of the nasal sac by a separate medial wall.

In *V. monitor* the lateral recess reaches its greatest development, and is completely cut off from the rest of the nasal sac for most of its length by a separate medial wall and floor (Text-figs 4I, J, 5A, B and 10B, C). The extent of the concha inside the main part of the nasal capsule is relatively small, it continues laterally, however, and runs down most of the length of the lateral recess capsule, to the medial wall of which it is attached (Text-figs 4I, J, 5A, B and 10B). As the roof of the recess capsule is only partly complete the anterior part of the concha is exposed from above (Text-fig 1A).

The lateral recess capsule may be regarded as a continuation of the main nasal capsule bent round laterally, so that the medial wall of the former is continuous with the lateral wall of the latter, which in this region is derived from paranasal cartilage. It therefore seems probable that the greater part of the recess capsule is of paranasal origin, with perhaps a dorsal plate contribution to the region behind the foramen epiphaniale. The relations of the paranasal cartilage and concha to the nasal sac in *V. salvator* and *monitor* are shown diagrammatically in Text-fig 10A, B.

The floor of the nasal capsule, in contrast with the roof, is very incomplete. Anteriorly, three small nodules of cartilage lie beneath the front edge of the anterior cupola (Text-figs 2A, 4B). Towards the midline the rostral process sweeps backwards and laterally to become continuous with the lamina transversalis anterior. The latter structure does not join the parietotectal cartilage to form a zona annularis, but runs backwards for some distance to a free posterior tip. The cartilage of Jacobson's organ projects upwards from the lamina transversalis (Text-figs 4D, E, 9A).

Pratt has stated that paraseptal and ectochoanal cartilages are absent in *Varanus*. Examination of further material, however, indicates that these are present in the usual saurian position. Both cartilages are more extensive in *V. monitor* than in *V. salvator* and *V. niloticus*. Near its root the paraseptal cartilage is much swollen and fragmented to form a cartilaginous network through which the vomeronasal nerve bundles pass on their way backwards from Jacobson's organ (Text-figs 3, 9B). The ectochoanal cartilage is directed medially and extends beneath the swollen part of the paraseptal, partly enclosing the fenestra vomeronasalis interna (Text-fig 9A). Above the ectochoanal cartilage and lamina transversalis anterior, the side wall of the nasal capsule is bent round and lies beneath the lateral part of the anterior chamber of the nasal sac (Text-figs 4F, G) so that the floor of the capsule is duplicated in this region.

Behind the level of the ectochoanal cartilage, the floor of the nasal capsule is absent, so that the fenestra endochoanalis is very extensive. In the complete skull, however, this cavity is partly closed by the vomer and the palatal process of the maxilla. The floor of the lateral recess capsule is continuous with the paranasal cartilage, the interpretation of this region has already been discussed. The lateral recess capsule lies against the concave inner aspects of the maxilla and prefrontal, and

is separated from the inwardly projecting maxillary shelf by the lachrymal ducts (Text-figs 4I, J, 5A, B)

The postnasal wall (Jarvik 1942 *planum antorbitale*, other writers) passes from the transverse segment connecting the capsule of the main nasal sac with that of the lateral recess to the edge of the nasal septum. It is in contact with the latter, but is not fused with it. Near the septum its lower edge is continuous with a small anteriorly directed process, which, by analogy with other forms, probably represents a detached caudal rudiment of the paraseptal cartilage (Text-figs 2A, 5E). The anterior face of the postnasal wall is slightly folded, accommodating small extensions from the antorbital space of the nasal sac. Behind the postnasal wall the prefrontal swings medially to form a rigid bony wall at the back of the nasal capsule (Text-fig 6)

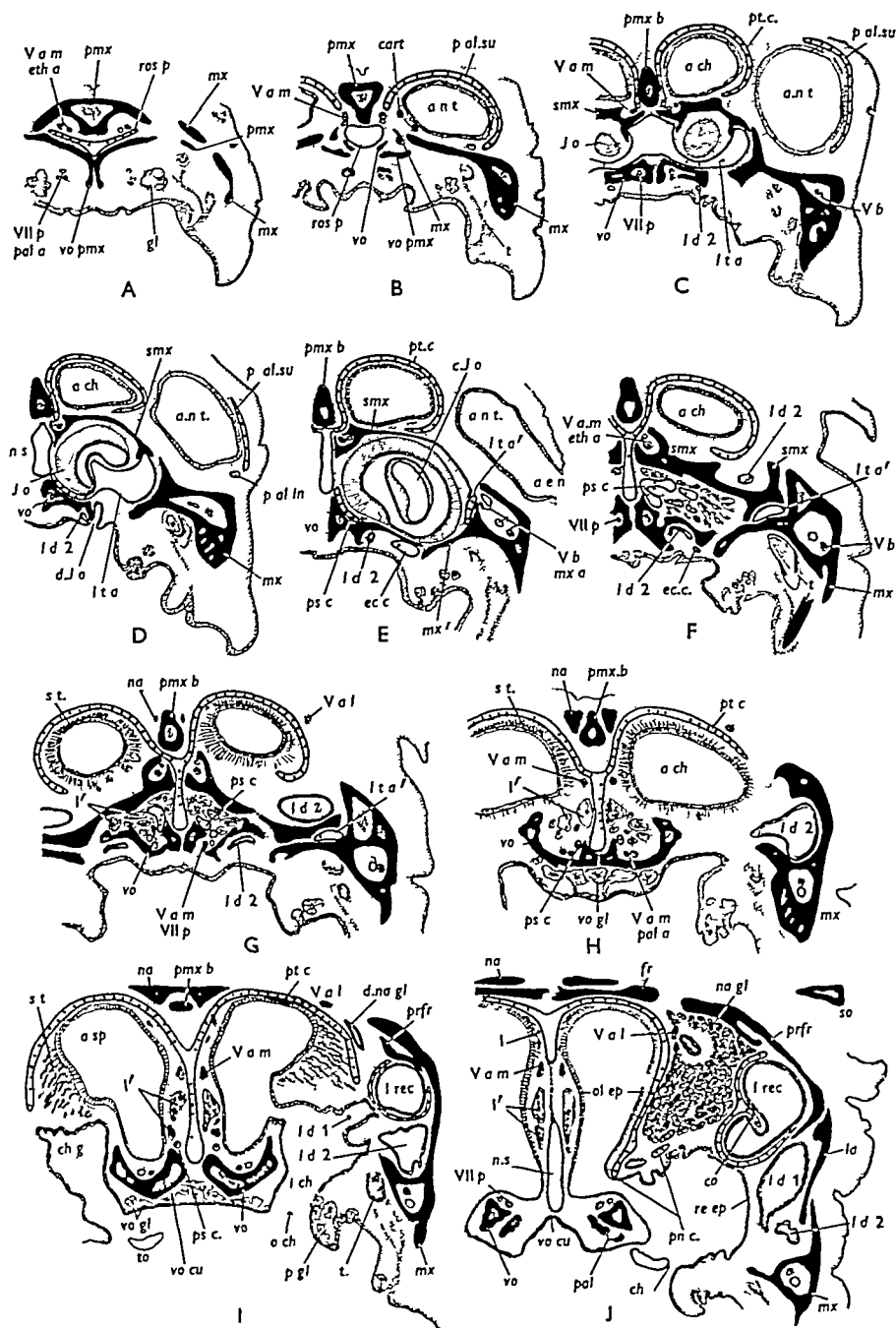
(3) *Nasal sac*

My reconstruction of the nasal sac of *V. monitor* (Text-fig 10D) agrees closely with those shown by Wegner (1922) and Pratt (1948). The anterior external nostril is longer and situated relatively farther forward in this species than in *V. niloticus* and *V. salvator* (Text-fig 10A, B). The anterior nasal tube is also more elongated and curved in *V. monitor*. It is possible that these differences have an adaptive significance, for *V. monitor* is a partly deserticolous form, whereas the other two species have amphibious tendencies (see Wegner, 1922; Mertens, 1942).

The anterior nasal tube passes medially to become continuous with the very elongated anterior chamber of the nasal sac. The latter is partly surrounded by spongy sinusoidal tissue (Text-fig 4G-I). Bruner (1907) has shown that this tissue is present in many lizards, and suggests that its intumescence provides a mechanism for closing the nasal passages without interfering with the other functions of the head. The anterior chamber leads backwards into the olfactory chamber, the point of entry of the nasal gland duct indicating the region of transition between the two. The most anterior part of the olfactory chamber is known as the anterior space (Beecker, 1903), and extends ventrally, coming into relationship with the front of the choanal groove (Text-fig 4I). The anterior space passes back into the conchal zone which is divided by the projecting concha into dorsal and ventral parts (Text-figs 4J, 5A, B). The ventral part of the conchal zone leads downwards through the opening of the inner choana into the choanal tube, which communicates with the mouth at the outer choana (Text-fig 10C). Behind the choanal region the nasal sac ends in a small blind pocket, the antorbital space (Text-fig 5D, E).

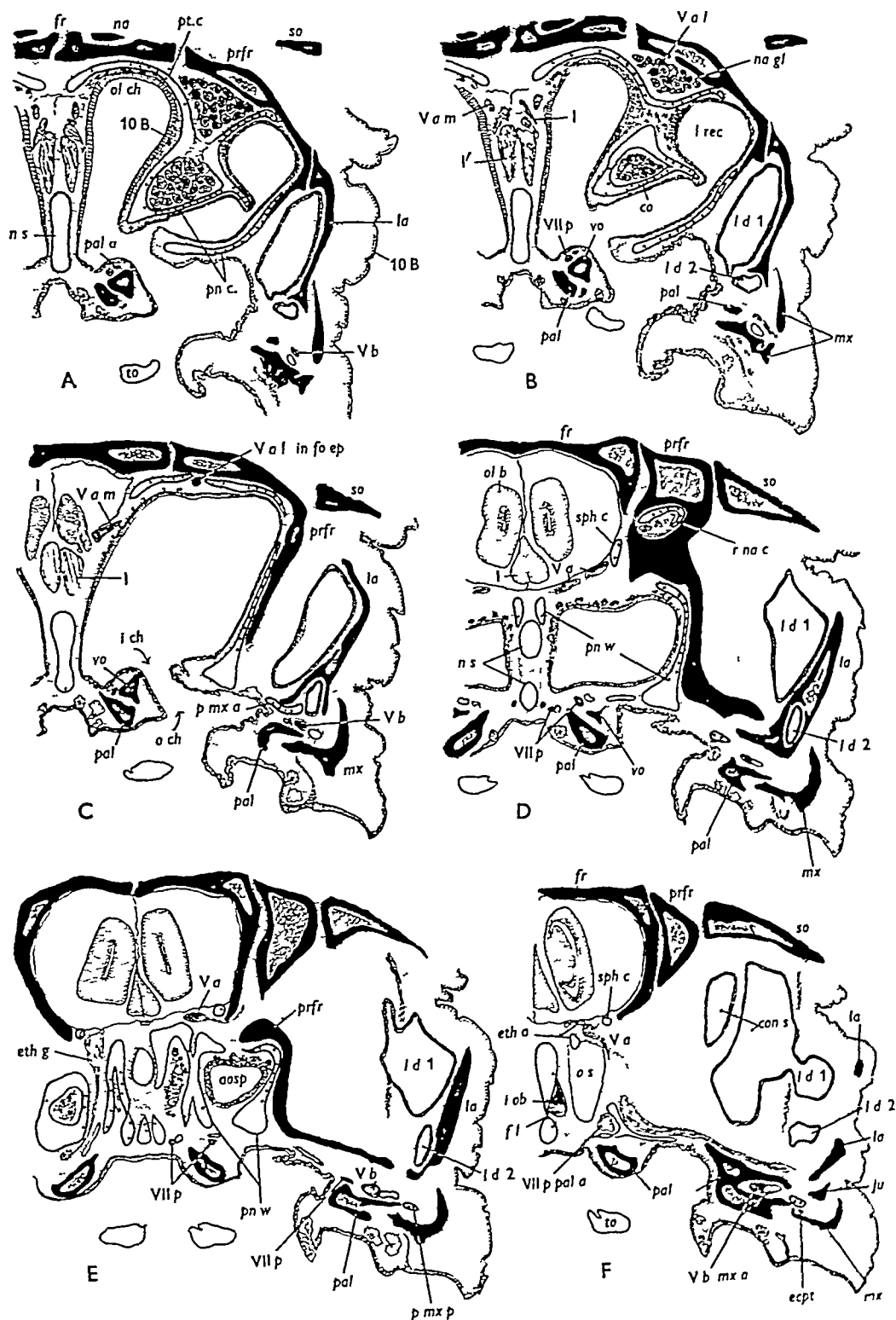
The lateral recess of the nasal sac communicates posteriorly with the main olfactory chamber (Text-fig 5A, B) and may be regarded as a continuation of it. The variations in the extent of the lateral recess in the different species of *Varanus* studied have already been described, it seems possible that these may also be related to differences in habits.

The anterior chamber is lined by squamous stratified epithelium which continues back on to the roof of the anterior space. The floor of the latter, however, is covered by ciliated epithelium of respiratory type and this extends backwards over the walls of the ventral conchal zone to the region of the choanal tube. Here the respiratory epithelium is replaced by the stratified squamous epithelium of the palate (Text-



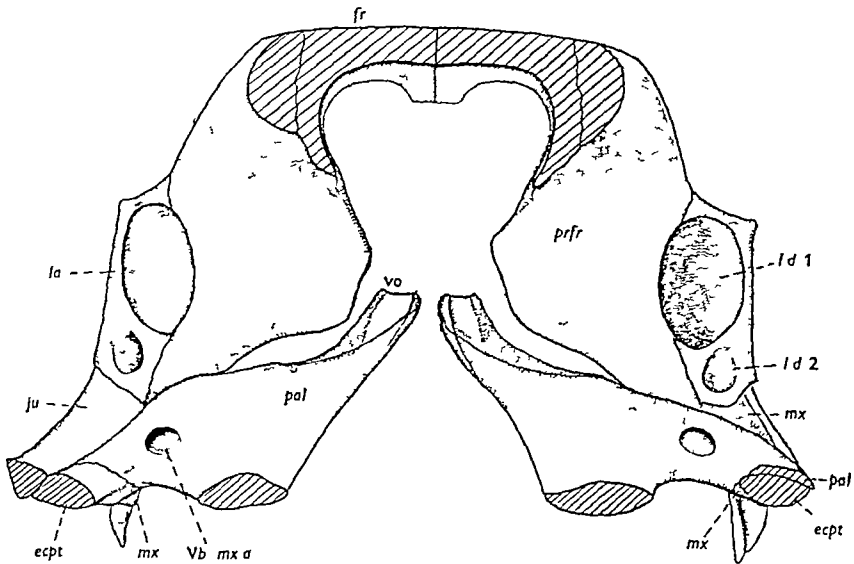
Text-fig 4

Text figs 4, 5 *Varanus monitor*, subadult Transverse sections through snout at levels shown in Text figs 1-3 ($\times 11$)



Text fig 5 (For explanation see p 125)

fig 10C) The sensory olfactory epithelium of *Varanus* is more limited in extent than in many other lizards. It is apparently confined to the dorsal surface of the conchal projection and to the roof and medial wall of the conchal zone, giving way to respiratory epithelium medially, above the inner choana (Text-fig 10C). The olfactory fibres arise from the sensory cells of the olfactory chamber and pass medially until they reach the side of the nasal septum. They become arranged into fibre bundles which run back dorsal to the vomeronasal nerves to enter the tip of the olfactory bulb (Text-figs 4I, J, 5A, B).



Text fig 6 *Varanus niloticus*, subadult. Transverse section of bony snout just in front of orbits, seen from behind. (Drawn from a dried skull.) The canal for lachrymal duct 2 penetrates the lachrymal bone, that for *ld 1* passes between the lachrymal and prefrontal. Cf. Text fig 5E, F for relationships of non bony structures. The jugal is missing on the right side. ($\times 6$)

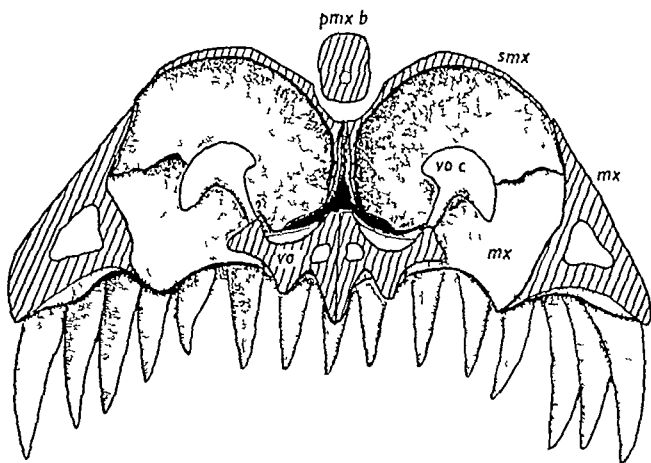
The appearance of the epithelium lining the lateral recess (Pl 1D) varies considerably in different places. At the tip of the concha there is a small patch of respiratory type epithelium containing mucous cells. The remainder of the recess is lined by epithelium of transitional type which on the dorsal surface of the concha approaches sensory olfactory epithelium in appearance. The sensory nature of this area might be expected if the dorsal part of the recess is regarded as a continuation of the main dorsal conchal zone. No nerve fibres were, however, observed to arise from the epithelium of this or any other part of the lateral recess.

(4) Organ of Jacobson

The vomeronasal organs (Text-figs 4D, E, 8, Pl 1C) are relatively larger than in most lizards. Experimental evidence based on other squamate types (e.g. Noble & Kumpf, 1936) has shown that odorous particles are conveyed to the duct of Jacobson's organ or to the neighbouring parts of the palate by the tips of the tongue. The

presence of a long bifid retractile tongue in *Varanus*, together with the large size of Jacobson's organs, suggests that the vomeronasal sense plays as important a part in the behaviour of these lizards as has been shown to be the case in snakes

In their general morphology the vomeronasal organs of *Varanus* conform to the usual squamate pattern as described by Pratt. Each organ possesses a large dorsal dome lined by sensory epithelium, and a mushroom body which is invaginated into the lumen from in front and below. This structure is covered by non-sensory ciliated epithelium, beneath which is a layer of rather dense connective tissue. This is supported by the cartilage of Jacobson's organ which projects upwards and backwards from the lamina transversalis anterior. The cartilage in turn rests on the vomerine concha which is carried on a stalk from the dorsal surface of the vomer (Text-figs 7, 8 and 9)

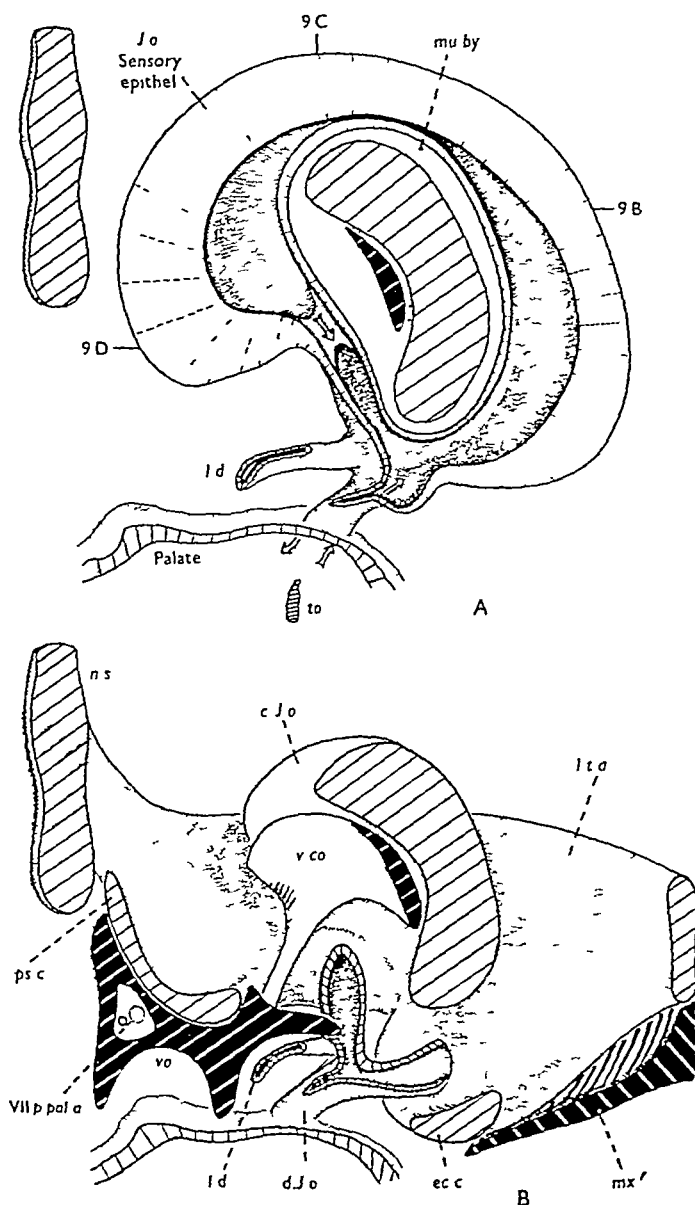


Text fig 7 *Varanus niloticus*, subadult. Anterior part of bony snout in transverse section seen from behind, showing chamber for Jacobson's organ. (Drawn from a dried skull.) The vomerine concha (vo c) supports the mushroom body of Jacobson's organ. Cf Pl 1C and Text figs 4E, 8 for relationships of non bony structures. ($\times 6$)

The spiral duct of Jacobson's organ (Text-figs 8, 9) has a crescentic opening into the lumen beneath the posterior part of the mushroom body, and passes downwards and forwards to lead into the mouth. The lachrymal duct opens into its medial aspect as it traverses the tissues of the superficial palate. The existence of a ciliary mechanism by which odorous particles are carried into the lumen of Jacobson's organ along the lateral aspect of its duct, over the mushroom body and back into the mouth down the medial aspect of the duct has been suggested by Pratt. The direction of this one-way circuit is indicated by arrows in Text-fig 8A. Since the duct of Jacobson's organ is lined by stratified epithelium, it would seem that odorous particles would have to be inserted into the lumen of the organ by the tongue tips before they could come under the influence of the cilia on the mushroom body.

The vomeronasal nerves are more numerous than the olfactory nerves. Their fibres arise from the sensory epithelium of the dorsal dome of Jacobson's organ and pass back over the concave upper surface of the vomer, between this bone and the septomaxilla. In this part of their course the fibres traverse the cartilaginous network formed by the swollen fragmented parts of the paraseptal cartilage (Text-

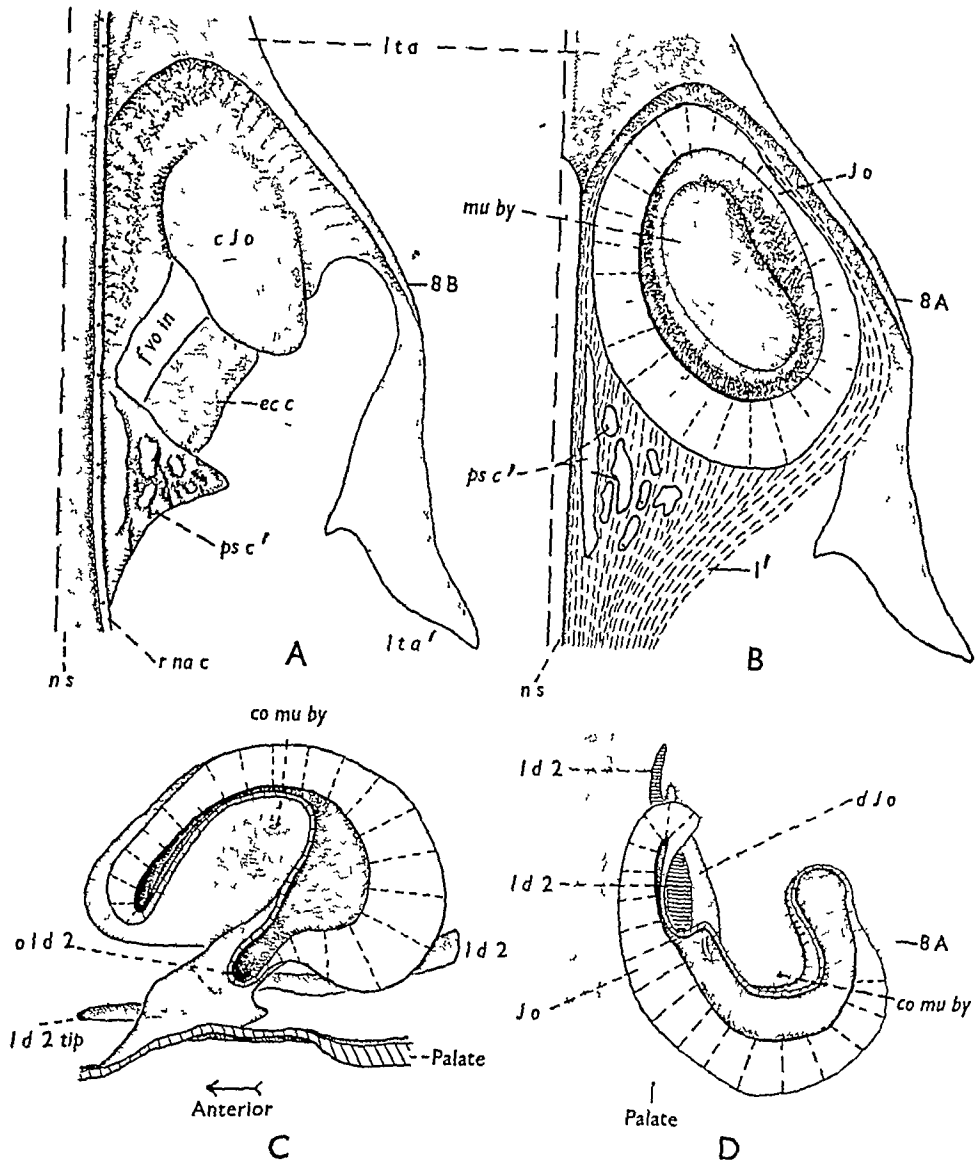
figs 4F, 9B) The nerves then become arranged into several large bundles which run back along the side of the nasal septum to reach the accessory olfactory bulb. The vomeronasal nerves, especially near their origin from Jacobson's organ, are richly



Text fig 8 A *Varanus monitor*, subadult. Reconstruction of right organ of Jacobson seen in transverse section from behind. Level of section shown in Text-fig 9B D. Arrows indicate probable direction of flow of particles. B *I monitor*. Reconstruction of cartilaginous capsule and duct of Jacobson's organ seen in transverse section from behind. Level of section as in Text fig 8A. level also shown in Text fig 9A. Some bones are included. The lachrymal duct *ld* figured is (*ld*) 2. (A B, $\times 50$)

vascularized, each of the main fibre bundles containing a dozen or more visible arterioles in any single cross-section

Each organ of Jacobson is almost completely surrounded by a skeletal investment from which it is separated by a layer of loose connective tissue containing many



Text fig 9 A *Varanus monitor*, subadult Reconstruction of cartilaginous capsule of Jacobson's organ seen from above after removal of roof of nasal capsule (cut edge shown) B *V monitor* Reconstruction of right organ of Jacobson and cartilaginous capsule seen in horizontal section from above at level shown in Text fig 8A The mushroom body is shown intact C *V monitor* Reconstruction of right organ of Jacobson seen in longitudinal section from lateral side Level of section shown in Text fig 8A Cartilage not included D *V monitor* Reconstruction of right organ of Jacobson seen in horizontal section from above at level shown in Text fig 8A Cartilage not included (All / 23)

blood-vessels This investment consists of an inner cartilaginous and an outer bony capsule (Text-figs 7, 8 and 9) The cartilaginous capsule is incomplete dorsally Its floor is formed by the hollowed posterior edge of the lamina transversalis anterior

with its projecting cartilage of Jacobson's organ, and farther back by the paraseptal and ectochoanal cartilages which extend posteriorly from the lamina. The latter cartilage projects downwards outside the bony capsule, passing between the vomer and the palatal process of the maxilla, and coming to lie in the superficial palate (Text-fig 4E, F). The medial wall is formed by the nasal septum, while an incomplete lateral wall is provided by the free lateral extension of the lamina transversalis anterior.

The bony capsule (Text-fig 7) is more complete, and encloses the organ of Jacobson and its cartilaginous capsule. The septomaxilla arches across the dorsal dome of the organ, separating it from the floor of the nasal sac. Laterally the septomaxilla is in contact with the vertical part of the maxilla, but medially and in front there is a gap between the septomaxilla and the dorsal surface of the vomer which is occupied by the nasal septum and the lamina transversalis anterior. These pass forwards out of the bony capsule to become continuous with the rostral process. At the posterior aspect of the bony capsule there is also a small vacuity between the septomaxilla and vomer through which pass the vomeronasal nerves.

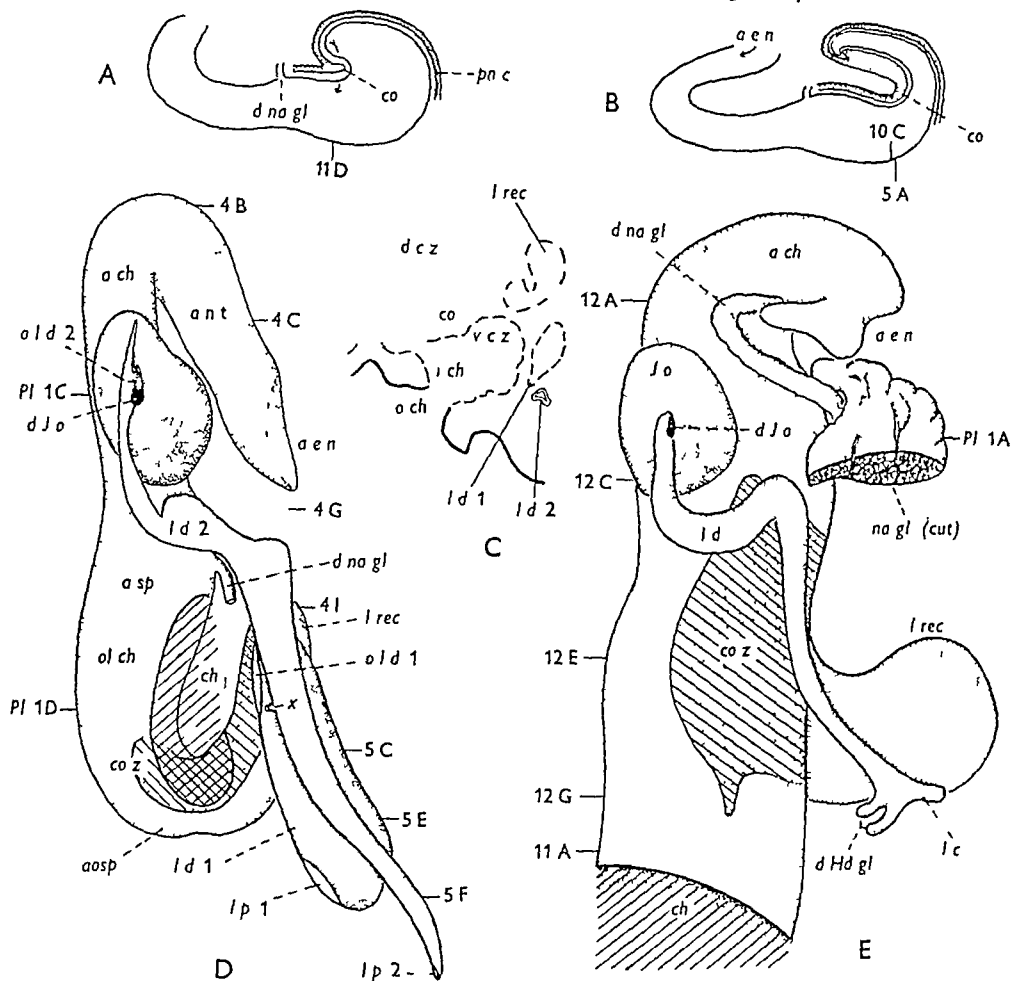
The floor of the capsule is formed by the lateral extension from the vomer and the palatal process of the maxilla. The duct of Jacobson's organ passes down between these structures (Text-fig 8B) through the fenestra vomeronasalis externa. Posterior to this level the lateral margin of the vomer and the maxillary palatal processes come into apposition so that the fenestra vomeronasalis is completely separated from the fenestra exochoanalis. This feature will receive further attention when the palate of *Varanus* is compared with that of other types.

(5) *Lachrymal apparatus*

V. monitor (and also probably *V. salvator* and *V. nloticus*, though the condition of the sections in this region made it impossible to confirm this with certainty) differs from all other lizards examined in that there are two separate lachrymal ducts on each side. Each duct arises at a separate punctum from the medial aspect of the lower eyelid, the punctum for the upper of the two ducts (lachrymal duct 1) is placed considerably in front of that for the lower duct (*ld* 2) (Text-fig 5F, Bellairs & Boyd, 1947). *ld* 1 is wider and much shorter than *ld* 2, it passes forward to enter the snout through a foramen between the prefrontal and lachrymal bones (Text-fig 6) and passes into an elongated gutter which opens into the ventral conchal zone of the nasal sac, above the inner choana (Text-figs 4I-J, 5A-F, Pl 1D). From its origin to about the level where it enters its bony canal, *ld* 1 is lined by stratified epithelium which is continuous with that of the palpebral conjunctiva, in the anterior part of its course, however, its epithelium is of ciliated respiratory type, similar to that of the ventral conchal zone into which the duct opens.

Lachrymal duct 2 passes back beneath the punctum for *ld* 1 (Text-fig 5F) and enters the snout through a foramen in the lachrymal bone (Text-fig 7). It then runs forwards on the inner surface of the vertical part of the maxilla, resting on the bony shelf formed by the maxillary palatal process (Text-fig 4H-J). In this situation it reaches a level a little posterior to Jacobson's organ (Text-fig 4H), and then curves sharply inwards and downwards, passing transversely across the concave posterior border of the septomaxilla to open into the medial aspect of the duct of Jacobson's

organ The angular part of the duct shows a marked dilatation which bulges forwards on to the dorsal surface of the septomaxilla (Text-figs 4F,G, 10D) Anterior to this level the diameter of the duct undergoes marked reduction, its opening into the duct of Jacobson's organ is elongated in the antero-posterior direction but is compressed from above downwards, so that its orifice has the form of a long narrow slit (Pl 1D, Text-figs 9C, 10D) The lachrymal duct continues forwards for some distance anterior to the level at which it opens into the duct of Jacobson's organ, ending blindly about the level of the front of the organ (Text-fig 1C)



Text fig 10 A, B Diagrams showing horizontal section of nasal sac in (A) *Varanus salvator* (plane of section shown in Text fig 11D), and (B) *V. monitor* (plane of section shown in Text fig 5A) (Partly based on personal communication from C W M Pratt) Conditions in these two species illustrate the development of the lateral recess and the corresponding changes in the concha and paranasal cartilage (stippled) C *V. monitor* Diagram showing different types of epithelium lining nasal sac etc, at level of Text fig 4J Stratified epithelium of palate shown in thick black lines, sensory epithelium over dorsal conchal zone (*d cz*) in dots, ciliated epithelium with mucous cells (respiratory type) over ventral conchal zone (*v cz*), upper part of choanal tube, tip of lateral recess concha and lachrymal duct 1, shown by interrupted lines, transitional epithelium with cilia but few mucous cells over most of lateral recess, shown by interrupted lines and dots, columnar non ciliated epithelium (*ld 2* only) in double lines D, E Diagrammatic reconstruction of nasal sac, organ of Jacobson and lachrymal ducts seen from below in (D) *V. monitor* and (E) *Xenopeltis unicolor* Extent of choanal tube is indicated by slanting lines, conchal zone (*co z*) shown by slanting lines and dots Levels of some sections in Text figs 4, 5, 11, and 12 and in Pl 1A,C,D are shown

At one point in its course, below the anterior opening of the upper duct, *ld* 2 communicates with the ventral conchal zone by means of a short transverse channel (Pl 1D), this channel has a small tortuous diverticulum leading from it. In this region a small invagination of epithelium and connective tissue projects into the main lumen of the duct. The significance of these features is obscure.

In the anterior part of its course *ld* 2 is lined by stratified epithelium. Around the level of the front of the lateral recess this is replaced by columnar non-ciliated epithelium which becomes continuous with the respiratory epithelium of the ventral conchal zone at the transverse communication (Pl 1D). In the posterior part of its course behind the lachrymal foramen, the epithelium again changes to the stratified type, as in the posterior part of *ld* 1.

The curious relationship of the two lachrymal ducts to the prefrontal and lachrymal bones (Text-fig 6) was first described by Mertens (1942), and is apparently constant for all species of *Varanus*. In all species the upper duct (*ld* 1) is wider than the lower, but the relative disparity in size of the two ducts varies considerably.

(6) Glands and teeth

The nasal (lateral nasal, Fahrenholz, 1937) gland is smaller than in many lizards and is situated in the aditus conchae, between the paranasal cartilage and the medial wall of the lateral recess capsule. Its duct leads forwards, hooking medially round the posterior of the two projections from the capsule roof, to open into the nasal sac around the level at which the anterior chamber becomes continuous with the anterior space (Text-figs 4I, J, 5A, B and 10D). Its acini are mainly mucous in character.

The distribution of the palatal glands follows the usual saurian pattern (Fahrenholz, 1937). A median vomerine group of glands lies along the lower border of the vomerine cushion, while the more scattered acini of the palatine group are situated farther laterally (Text-fig 4I). The superior labial glands are represented only by a few isolated acini along the medial aspect of the dental lamina. The glands of the palate appear to be mainly serous in character.

The pleurodont teeth of *Varanus* vary in appearance, depending on the age and species of the individual. In many cases they are sharp and recurved. In mature specimens, particularly of *V niloticus* and *V exanthematicus*, they may be blunt and rounded (Mertens, 1942). It is possible that the diet, which ranges from small vertebrates to insects and crabs (Cowles, 1930), may also be associated with the form of the teeth. No study of the histology or mode of tooth succession was made, a review of the relevant literature is given by Peyer (1937).

(7) Nerves

The topography of the cranial nerves has been described by Fischer (1852) in *Varanus bengalensis* and by Watkinson (1906) in *V bivittatus*, while Bahl (1937) has enumerated the nerve foramina in the bony skull of *V monitor*. A more detailed account of the cranial nerves of a lizard has been given by Willard (1915) for the iguanid *Anolis carolinensis*. My own findings largely confirm those of the workers cited but in the absence of selectively stained material it has not been possible to investigate the distribution of nerve endings, or ganglionic connexions. The following account is based mainly on serial sections of *V monitor*.

Apart from the nerves of special sense, the snout receives its innervation from three sources the ophthalmic and maxillary divisions of the trigeminal, and the palatine branch of the facial nerve. The two branches of the fifth nerve probably carry fibres which are mainly somatic sensory in nature, the extensive communication between these two nerves and the palatine nerve, however, renders it likely that some autonomic fibres are also distributed with the two trigeminal branches. The palatine nerve is probably composed in the main of pre- and post-ganglionic parasympathetic fibres which are distributed to the glands and blood vessels of the snout. Sympathetic and visceral afferent fibres may also be present (Willard, 1915, Adams, 1942).

The ophthalmic nerve passes along the medial aspect of the orbit on either side of the interorbital septum, and enters the snout through the orbitonasal fissure, between the septum and the sphenethmoid commissure (Text-fig 5F), where it becomes the ethmoidal nerve. As it lies in the fissure it possesses a swelling in which many ganglion cells can be seen. This swelling corresponds with the ethmoidal ganglion described by Willard in *Anolis*, and communicates with the palatine nerve by a band of fibres which runs vertically downwards behind the postnasal wall (Text-fig 5E).

Slightly anterior to the ethmoidal ganglion the nerve divides into its lateral and medial rami (Text-fig 5D). The lateral ramus passes forwards beneath the roof of the nasal capsule for a short distance, and then leaves the capsule through the foramen epiphaniale (Text-fig 5C). It then breaks up into numerous branches among the acini of the nasal gland (Text-fig 5B, etc.) and probably supplies the latter (see Gaupp, 1888) as well as the skin and tissues of the snout.

The medial branch of the ethmoidal nerve passes forwards at the side of the nasal septum where it is closely related to the olfactory and vomeronasal nerves (Text-figs 4, 5A-C). It enters a groove along the medial edge of the septomaxilla and runs forwards in this situation (Text-fig 4D-G), escaping through a foramen at the front of the bone (Text-fig 4B, C). Anterior to this level it lies between the rostral process and roof of the nasal capsule (Text-fig 4B), and in front of this again, between the rostral process and premaxilla, the slightly different condition in *V. salvator* has been described. The nerve then enters the posterior premaxillary foramen (of Bahl) and divides into dorsal and ventral branches which emerge from the dorsal and ventral premaxillary foramina respectively (Text-figs 1A, 2A) to supply the tip of the snout. No branches supplying the nasal sac or the organ of Jacobson could be identified, although the existence of such branches seems probable.

The maxillary nerve passes along the ventrolateral aspect of the orbit and enters the snout through a canal in the palatine bone (Text-figs 5F, 6). During its course through the orbital region it has two principal communications with the palatine nerve: one near the back of the orbit and another just anterior to the orbit. After the nerve has left the palatine canal (Text-fig 5E) it enters the maxillary canal after giving off several small branches which are probably distributed to the glands and epithelium of the palate. At intervals throughout its course in the maxillary canal the nerve supplies the teeth, and also sends branches laterally which pass through small foramina in the bone and are distributed to the side of the jaw. A few rami also pass medially through the palatal process of the maxilla to reach the palate. One rather large branch emerges from the easily recognizable anterior maxillary foramen (Bahl) which lies lateral to the fenestra vomeronasalis externa (Text-

fig 2A) Another large branch also passes upwards and medially through a foramen in the dorsal aspect of the maxilla (Text-figs 1A, 4E), and is apparently distributed to the anterior nasal tube. The main nerve trunk finally escapes through a foramen near the anterior extremity of the maxilla and breaks up into branches in the superficial tissues of the snout.

The palatine branch of the facial nerve leaves the parabasal canal and passes forwards beneath the orbit at first medial to the pterygoid and then dorsal and lateral to it. In this situation, a little behind the midorbital region, it communicates with the infraorbital (maxillary) nerve, and bears a swelling containing ganglion cells. This swelling corresponds in position with the palatine ganglion in *Anolis*, and with the sphenopalatine ganglion figured by Adams (1942) in *Lacerta viridis*. Anterior to this level the nerve continues above the palatine bone, sending branches to the superficial palate. At the front of the orbit the nerve swings medially and enters the snout near the lower edge of the interorbitonasal septum (Text-fig 5F). In this situation the nerve carries another ganglionic swelling which is connected with both the ethmoidal ganglion above and the maxillary nerve laterally, a small bundle of fibres joins the maxillary nerve and passes with it into the maxillary canal (Text-fig 5E). This anterior ganglion probably corresponds with the structure described by Haller & Hallerstein (1937) in *Anguis fragilis*. After giving off several lateral branches to the superficial palate the main trunk of the nerve passes forwards above the vomeropalatine articulation (Text-fig 5A-D) and then over the concave upper surface of the vomer (Text-fig 4G-I). In the region of Jacobson's organ it becomes completely enclosed in a canal in the medial part of the vomer, from which it sends at least one branch downwards through the bone to reach the superficial palate (Text-fig 4G), probably supplying the palatine glands and epithelium (Gaupp, 1888). Anteriorly the nerve leaves the vomerine canal and breaks up into fine branches in the premaxillary region of the superficial palate.

No fibres from the trigeminal (or facial) nerve supplying Jacobson's organ could be identified, nor have any been described by Watkinson, nor by Willard in *Anolis*. It seems likely, however, that Jacobson's organ receives some sensory trigeminal innervation, it is unfortunate that in *Anolis*, the only form of which selectively stained preparations have been studied, the organ of Jacobson is smaller and less conspicuous than in most lizards. The nervus terminalis could not be identified in *Varanus*.

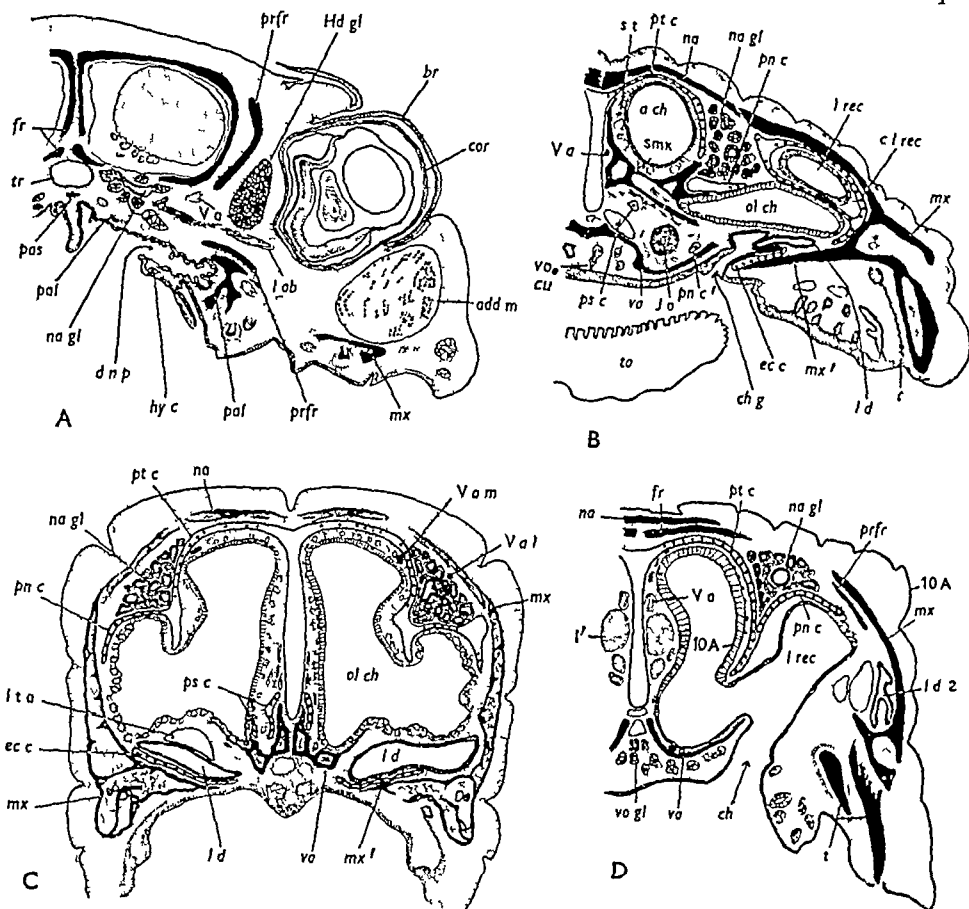
(8) Blood vessels

The vascular system of *V. ('Psammosaurus') griseus* was studied by Corti (1847), but he hardly deals with the vessels of the snout. Subsequent descriptions of the blood supply of the snout in lizards have been given by Bruner (1907), who dealt only with the veins, and by Hafferl (1933) in the Bolk Handbook, while O'Donoghue (1920) has described the condition in *Sphenodon*.

The arterial supply of the snout is derived from three main vessels which all stem ultimately from the internal carotid: the supraorbital, infraorbital and palatine arteries.

The supraorbital artery in *Varanus* seems to be smaller than in *Sphenodon*. It arises from the temporal artery behind the orbit and passes forwards above the eye, lying at first lateral to the taenia marginalis and then running downwards and medially on the lateral aspect of the downgrowth from the frontal bone. It enters

the snout through the orbitonasal fissure in proximity to the ethmoidal nerve, and becomes the ethmoidal artery (Text-fig 5F) The artery then breaks up into a large number of dorsal and ventral branches behind the postnasal wall which run forwards to supply the nasal sac and the spongy tissue around the anterior chamber One large ventral branch runs downwards and laterally to anastomose with the palatine



Text fig 11 A *Xenopeltis unicolor*, subadult Transverse section through orbital region at level indicated in Text figs 10, 13, showing ductus nasopharyngeus B *Tarentola mauretanica*, adult Transverse section of snout through posterior part of Jacobson's organ at level indicated in Text figs 1F, 2B, showing paleochoanate type of palate C *Tupinambis teguixin*, subadult Transverse section through snout behind Jacobson's organ at level indicated in Text fig 2C, showing incomplete neochoanate type of palate D *Varanus salvator*, subadult Transverse section through snout at level corresponding with that shown in Text fig 4J of *V. monitor* The lateral recess is not closed off in *V. salvator*, and *ld 1* is not seen at this level Plane of Text fig 10A shown (A, $\times 13.5$, B, $\times 18$, C, $\times 7$, D, $\times 9$)

and maxillary arteries (Text-fig 5E,F) Some of the dorsal branches appear to supply the tips of the olfactory bulbs as well as the olfactory nerves A large branch on each side passes forwards to enter the septomaxillary canal with the medial ethmoidal nerve, and is distributed with it (Text-fig 4F)

The infraorbital artery also arises from the temporal artery and runs forwards on the ventrolateral aspect of the orbit, near the infraorbital (maxillary) nerve At the front of the orbit it receives anastomotic connexions from the palatine and ethmoidal arteries and enters the maxillary canal with the maxillary nerve, being distributed

with it to the teeth, jaws, palate and sides of the snout and, through its dorsal branch, to the spongy tissue around the anterior chamber of the nasal sac

The palatine artery arises directly from the internal carotid and passes along the roof of the mouth near the palatine nerve. In the snout it sends numerous branches to supply the palatine glands and epithelium. A large branch on each side runs forwards to enter the vomerine canal with the palatine nerve. This sends many large branches to supply the organ of Jacobson and vomeronasal fibre bundles (Text-fig 4G, etc.)

The complicated system of venous sinuses in the saurian head has been thoroughly described by Bruner (1907) in *Lacerta agilis* and other forms. It was not possible to make any critical investigation of these structures in my own material. Bruner's observations show that almost all the venous drainage of the snout passes through an extensive series of nasal and palatine veins into the large orbital sinus (Text-fig 5F), and ultimately into the internal jugular veins.

COMPARISON WITH OTHER LIZARDS

A detailed survey of the saurian snout and the extensive literature concerned with it is beyond the scope of this account, it is only possible, therefore, to touch on those features which seem relevant to the affinities of *Varanus*, or appear of general morphological interest.

The curiously incomplete condition of the dermal skeleton of the varanid snout does not seem to occur in other living lizards. In most forms the fenestra exonarina anterior is relatively small, and is bounded medially and in front by the premaxilla, and laterally by the maxilla which conceals the septomaxilla almost entirely. Such a condition is present in the gecko *Tarentola mauritanica* (Text-fig 1F). In *Varanus* the maxilla does not conceal the septomaxilla in this way and the greater part of the latter bone is exposed in the huge fenestra exonarina when the nasal capsule has been removed (Text-fig 1A). The only forms which seem to approach the varanids in this respect are the Cretaceous mosasaurs. This region has been clearly described and figured by Camp (1940) in *Kolposaurus*, where the long slender premaxillary bar and the exposed septomaxillae are highly reminiscent of the varanid condition. The septomaxilla is, however, narrower and more elongated in the mosasaur, and it may be supposed that the bony chamber for Jacobson's organ was less spacious in the sea lizard than in *Varanus*.

The palate of *Varanus* is remarkable for the completeness of both its bony and superficial components. In an extensive study of the bony palate of Sauria, Lakjer (1927) has shown that three main varieties may be distinguished. In the first type the palate is only complete as far back as the anterior margin of the fenestra vomeronasalis externa which is confluent with the fenestra exchoanalis. This type (called by Lakjer palechoanate) is present in forms such as agamids, iguanids and geckos (e.g. *Tarentola*, Text-figs 2B, 11B). It is regarded as the most primitive variety of saurian palate, and approximates to the condition in *Sphenodon* and the early cosuchian reptiles. An advance on this type of palate is shown by the incomplete neochoanate condition which occurs in forms such as skinks, lacertids and lizards (e.g. *Tupinambis*, Text-figs 2C, 11C). Here the fenestra vomeronasalis externa is partly cut off from the fenestra exchoanalis by the palatal process of the

maxilla, which extends medially beneath the lateral margin of the vomer (Text-fig 11C) The final complete neochoanate condition is found only in the genus *Varanus*, where the palatal process of the maxilla is still more extensive, and actually contacts the vomer for a short distance behind the fenestra vomeronasalis, separating it from the fenestra exochoanalis (Text-figs 2A, 4E-G) *

These variations correspond more or less closely with the condition of the superficial palate, which forms the subject of a most extensive German literature (see Barge, 1937, for review) In the paleochoanate and incomplete neochoanate forms the choanal tube is continuous anteriorly with a long choanal groove which passes upwards towards the nasal sac, separating the median vomerine part of the palate (vomerine cushion see Text-fig 11B) from the lateral maxillary portion In many forms the lachrymal duct discharges into the choanal groove, but in others, such as *Varanus* (Pl 1C) and *Tupinambis* (Pl 1B) it opens directly into the duct of Jacobson's organ These relationships are to a large extent dependent upon the degree of fusion between the frontonasal and maxillary processes during ontogeny It is hoped that some account of the embryological processes involved, together with the numerous variations in this region which occur throughout the Sauria, will be given in greater detail elsewhere

Pratt (1948) has suggested that the presence of these choanal grooves provides a mechanism for the filling of Jacobson's organ, and that particles deposited in them by the tongue tips are carried forwards by ciliary action into the lumen of the organ In *Varanus* these choanal grooves are relatively short, and do not extend forwards to reach the duct of Jacobson's organ It is, therefore, assumed that the particles are inserted inside the orifice of the duct, or actually into the lumen of the organ This mechanism, which probably also occurs in snakes where the anatomical relationships are very similar, is reputed to be more effective than the method which is dependent on the action of cilia in the choanal grooves It may be noted that the long retractile tongue of *Varanus*, which has been regarded as suggesting affinities with snakes, is also present in the large American tend *Tupinambis* Although the bony palate of this form is incompletely neochoanate, the morphology of the superficial palate, with short choanal grooves and the lachrymal duct opening directly into the duct of the organ of Jacobson (Pl 1B) approaches the varanid condition A rather similar state is also present in some of the Amphisbaenidae, a group of specialized burrowing reptiles usually classified with the Sauria

The general appearance of the nasal capsule, with its elongated form, incomplete floor and (in *Varanus monitor*) enormous lateral recess bears a certain resemblance to the condition in *Eumeces quinquelineatus* as described by Rice (1920), and perhaps also to that in certain agamids (e.g. *Agama atricollis*, Pratt, 1948) It would be rash, however, to suggest that these features have any great systematic value in view of the striking interspecific differences within the genus *Varanus*

The long rostral process with its lateral wings extending forward of the nasal cupola, and the low anterior part of the nasal septum are unusual The absence of

* It may be noted that the small 'secondary palate' formed in this way differs from the condition in crocodiles and in mammals In these forms the secondary palate is derived entirely from the lateral bones (maxillae, palatines, etc.) which fuse in the midline, displacing the vomers upwards in relation to the nasal septum

a discrete foramen apicale in *V. monitor* and the relationships of this foramen in *V. salvator* are also noteworthy

Another feature of interest is the disposition of the paraseptal cartilage and the way in which the ectochoanal cartilage runs backwards and medially to extend beneath it. In many lizards (e.g. *Lacerta agilis*, Gaupp, 1900, *Eumeces quinquelineatus*, Rice, 1920) the ectochoanal cartilage projects backwards and outwards. Its inward direction in *Varanus* almost encloses the fenestra vomeronasalis interna, separating it from the fenestra endochoanalis in the same way as the vomeromaxillary contact separates the fenestra vomeronasalis externa from the fenestra exochoanalis.

The significance of the fragmentation of the paraseptal cartilage into a network surrounding the vomeronasal nerves is obscure. This feature was present in all the three species of *Varanus* studied, and also occurs in the genus *Gerrhosaurus* (Malan, 1941), in *Tupinambis teguixin*, and, to a lesser extent, in *Lacerta vivipara*.

COMPARISON WITH SNAKES

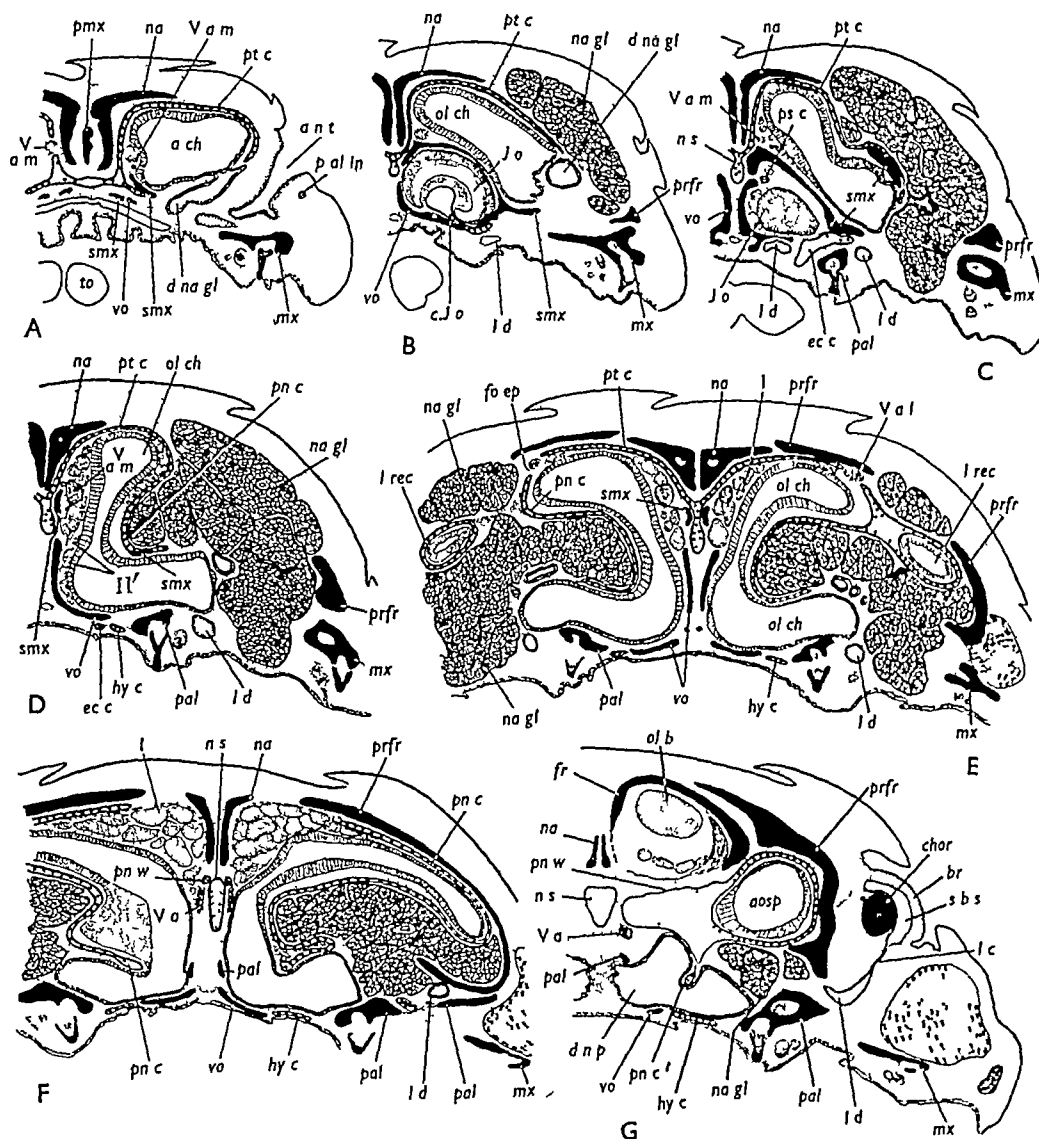
The Sunbeam snake, *Xenopeltis unicolor* Reinwardt (fam. Xenopeltidae), has been selected for comparison with *Varanus*. Two series of sections through the nasal region of this primitive and comparatively generalized ophidian have been available for study, for the specimens from which these have been prepared I am indebted to Dr Malcolm Smith, and to Mr M. W. F. Tweedie of the Raffles Museum, Singapore.

The snout of *Xenopeltis* is shorter and wider than is the case with most lizards, and contrasts particularly with the elongated snout of *Varanus*. The dorsal surface of the skull (Text-fig. 1B) shows a large fenestra exonarina anterior in which the septomaxilla is widely exposed. The relationships of the bones are so different from those in *Varanus*, however, that the latter feature can hardly be regarded as suggesting affinity between the two forms.

The bony palate of *Xenopeltis* (Text-fig. 13) differs even more strikingly from the saurian condition. The premaxillae form a rounded arcade which is in contact with the anterior tip of the maxilla on each side. This condition is found only among the most primitive snakes, and in more advanced forms such as the colubrids and vipers the premaxillae are much reduced and are not in contact with the maxillae. In all non-viperine snakes* the lateral extent of the maxilla is reduced and the bone has the form of a narrow rod which is separated from the vomer and palatine medially by a considerable gap in which the septomaxilla is widely exposed. The fenestra vomeronasalis externa lies between the septomaxilla laterally and the vomer medially, instead of between the maxilla and the vomer, as in *Varanus* and other lizards. A peculiar feature in *Xenopeltis* is the presence of a long slender process which runs forward from the main part of the palatine bone and lies above the vomer in the medial wall of the ductus nasopharyngeus (Text-figs. 11A, 12F, G and 13). The position of the fenestra exochoanalis in *Xenopeltis*, as in snakes generally, is very different from that in Sauria. In the latter the fenestra occupies a large part of the bony palate and is situated between the maxilla and the vomer, with the palatine behind (Text-fig. 2A, B, C). In snakes the fenestra is directed backwards from above the posterior part of the vomer (Text-fig. 11A) and is hardly visible from the ventral

* In the vipers the maxilla is short and highly specialized in association with the mechanism of fang erection.

aspect The fenestra vomeronasalis externa is therefore separated by the greater part of the length of the vomer from the fenestra exochoanalis. An extensive bony 'secondary palate' is therefore present, but this lies only beneath the medial part of the nasal sac which extends laterally across the gap between the vomer and the palatine

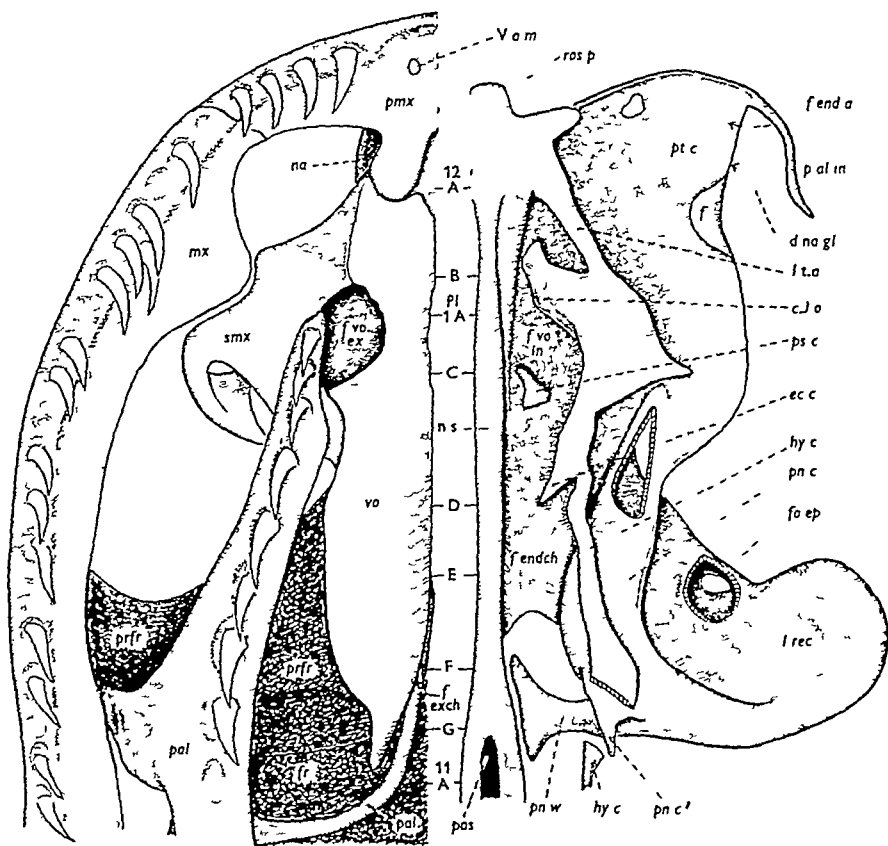


Text fig 12 *Xenopeltis unicolor*, subadult. Transverse sections through snout at levels shown in Text figs 10E and 13 ($\times 15$)

The appearance of the nasal sac in *Xenopeltis* with its short anterior nasal tube and poorly differentiated lateral recess is shown in Text-fig 10E. As in all snakes the choanal tube is drawn out into an elongated ductus nasopharyngeus which lies dorsal to the posterior part of the vomer (Text-fig 11A). A ductus nasopharyngeus is also present in many skinks and certain other lizards, but here the bony relationships are very different (see Lakjer, 1927, Barge, 1937).

The most striking similarity between *Varanus* and the snakes is shown by the

condition of Jacobson's organ, by the relationship of its duct to the lachrymal system, by the morphology of the superficial palate, and by the presence of a slender bifid tongue. These structures, together with the eye glands which provide the secretion in which the odorous particles deposited by the tongue tips are carried through Jacobson's organ, constitute a functional 'vomeronasal unit' which seems to have reached its greatest development among Squamata in the varanid lizards and the snakes. In the latter a single lachrymal duct only is present. It will be seen, however



Text fig 13 *Xenopeltis unicolor* subadult. Reconstruction of chondrocranium (right) and bones of snout (left) seen from below. (Bones drawn from dried skull.) Levels of section in Text-figs 11 and 12 are shown. Windows have been cut in the paranasal cartilage and lateral recess capsule and a segment of the hyopchoanal cartilage has been removed. ($\times 25$)

(Text-fig 10E), that the relationships of the anterior part of this structure, with its dilated angular portion behind the organ of Jacobson, and its direct opening into the duct of the latter organ, are essentially similar to those of the long lachrymal duct 2 of *Varanus*. The posterior (orbital) end of the lachrymal duct system is markedly different in *Varanus* and *Xenopeltis*, however, conditions in the former following the usual saurian pattern with the lachrymal puncta arising from the conjunctival space on the deep aspect of the lower eyelid (see Bellairs & Boyd, 1947)

The nasal capsule of *Xenopeltis* differs little from that of more advanced and 'typical' snakes such as *Natrix* (*Tropidonotus*) *natrix* (Backstrom, 1931). Characteristic ophidian features are the high anterior cupolae joined by a flattened rostral process which runs back into the low nasal septum, the long processus alaris inferior and the extensive conchal infolding which is exposed from below by the almost complete absence of the true capsule floor. The ectochoanal cartilage is connected with a long hypochoanal cartilage which runs back to support the ductus nasopharyngeus. As in snakes generally, the sphenethmoid commissures are absent.

Perhaps the only feature in the nasal capsule of *Xenopeltis* which can be regarded as primitive is the presence of a small nodule of cartilage on each side of the nasal septum behind the fenestra vomeronasalis interna (Text-figs 12C, 13). From its position and relationship to the vomeronasal nerves, this nodule appears to represent a rudiment of the paraseptal cartilage of lizards. No similar structure has been reported in other snakes, nor have I found anything resembling it in sections of other forms usually regarded as primitive (e.g. *Cylindrophis*, *Eryx*, *Constrictor*).

DISCUSSION

In assessing the affinities of any form it is always a matter of difficulty to distinguish between those resemblances with other types which are indicative of true phylogenetic relationship, and those which result from parallel evolution. This is particularly the case with a group like the Squamata, where extensive parallel evolution has occurred in association with a host of divergent specialized trends. It is, therefore, unwise to attempt even a provisional assessment of the relationships of *Varanus* without some consideration of a wider range of characters than has been described in this account.

The striking differences between the snout of *Varanus* and that of other lizards can be matched with certain peculiarities shown by other regions of the body. The presence of cartilage canals in the epiphyses of the long bones, a feature unique among reptiles (Haines, 1941), together with other characters listed by Camp (1923) are suggestive of a long evolutionary history distinct from other living saurian families. Relationship with the mosasaurs, however, is indicated by numerous features of cranial morphology (see Camp, 1940).

In spite of its peculiarities, the basic morphology of the varanid snout, as exemplified by such structures as the bony palate and nasal capsule, are essentially saurian rather than ophidian in nature. Apart from the slightly ophidian appearance of the anterior part of the nasal septum and its relationship with the nasal cupolae and site of exit of the ethmoidal nerves, the few absolutely characteristic features of the ophidian nasal capsule are not shown, or even approached, in *Varanus*. Such typical ophidian features are the long processus alaris inferior, the presence of the hypochoanal cartilage, and the absence (or marked reduction) of the paraseptal cartilage.

The undoubted resemblances between the varanid superficial palate and vomeronasal apparatus do not seem of great phylogenetic significance. The fact that these resemblances involve the soft parts only, and are superimposed upon skeletal structures of a very different type, is suggestive of parallel evolution associated with the development of a highly efficient vomeronasal sense. The similarity between the

omeronasal organs and palate of *Varanus* and those of at least two very distinct squamate genera, *Amphisbaena* and *Tupinambis*, lends further support to this view.

An investigation of the endocranial brain-case in reptiles shows that one of the most clearly marked distinctions between the cranial anatomy of lizards and snakes lies in the disposition of the trabeculae in the orbital region, and in the presence or absence of sphenolateral cartilages. The varanid brain-case with its high interorbital septum and fenestrated sphenolateral is essentially similar to that of such generalized lizards as *Eumeces* and *Lacerta*, and is in striking contrast with the platytrabic condition of snakes where the sphenolateral cartilages are absent in the adult state. Other features of the varanid skull such as the presence of the lachrymal and jugal bones and the structure of the ear seem to show that, on grounds of cranial morphology at least, there is no reason to regard *Varanus* as having any specially close affinities with the snakes. Indeed, certain other saurian groups appear to show a closer resemblance to snakes in many important respects (see Bellairs, 1949).

On the classification devised by Camp (1923) the Varanidae appear as the closest living relatives of snakes, and this concept has been expressed more emphatically by Romer (1945), who writes that the snakes are 'obviously derived from varanid types'. Camp's original view seems to have been mainly based on the structure of the hemipenes and vertebrae, together with certain cranial features, which, as I hope to show elsewhere, are open to a different interpretation. I do not feel that this view has been adequately substantiated by the study of cranial morphology, which has so far failed to reveal any significant resemblances between *Varanus* and the snakes which cannot as readily be explained on grounds of parallel evolution. It is therefore suggested that further evidence is required before a specially close relationship between the Varanidae and the snakes can be established.

SUMMARY

1 The anatomy of the snout of *Varanus* is described, largely from serial sections of the species *monitor*, *salvator* and *niloticus*. The nasal capsule, nasal and vomeronasal organs, lachrymal apparatus and palate are described in some detail, and an account is also given of some features of the bony snout, and the distribution of the main nerves and arteries. The terminology devised by Jarvik (1942) for the different parts of the ethmoidal region of anamniotes has been applied and extended. The most important features shown by *Varanus* are listed below.

2 The nasal capsule is incompletely covered by dermal bones on the dorsal surface of the snout. The fenestra exonarina anterior is very large, with the septomaxilla widely exposed in it. (Condition approached by mosasaurs.)

3 The nasal capsule is elongated with an unfenestrated roof. The nasal septum ends anteriorly in a flattened rostral process which extends forwards in front of the cupolae. The floor of the nasal capsule is largely incomplete and the zona annularis is absent. Paraseptal and ectochoanal cartilages are present and almost enclose the fenestra vomeronasalis interna. There is an extensive separate capsule for the lateral recess of the nasal sac in *V. monitor*. (This feature is present in some other lizards such as *Eumeces*, but is less well developed in *Varanus salvator* and *V. niloticus*.)

4 The anterior nasal tube is long and curved and the anterior chamber of the nasal sac is elongated. The olfactory chamber is limited in extent. The nasal concha

extends into the lateral recess in *V monitor* only. The choanal opening is limited to the posterior part of the nasal sac, but is not drawn out to form a ductus nasopharyngeus as in some lizards and all snakes.

5 The organ of Jacobson is very large and its duct opens into the mouth and receives the anterior orifice of lachrymal duct 2.

6 There are two separate lachrymal ducts on each side. Lachrymal duct 1 (arising from the superior punctum) is short and discharges into the olfactory chamber of the nasal sac after passing between the prefrontal and lachrymal bones. Lachrymal duct 2 is long and passes through a foramen in the lachrymal bone to discharge into the medial aspect of the duct of the organ of Jacobson. The relations of lachrymal duct 2 somewhat resemble those of the single lachrymal duct of snakes.

7 The bony palate is completely neochoanate, with the fenestra vomeronasalis externa separated from the fenestra exochoanalis by the vomeromaxillary contact. The superficial palate is remarkably complete, with short choanal grooves.

8 A brief description of some of the features of the snout of the primitive snake *Xenopeltis unicolor* is given. Except for the presence of a nodule interpreted as a rudimentary paraseptal cartilage, conditions are very similar to those in more advanced snakes such as *Natrix*. The most striking differences from the saurian condition are shown by the morphology of the bony snout, especially the palate, and by certain features of the nasal capsule.

9 A comparison between *Varanus* and other lizards suggests that its isolated systematic position within the suborder Sauria is fully in accord with the findings described.

10 A comparison with snakes shows that, in spite of its peculiarities, the genus *Varanus* conforms in all essential respects to the saurian rather than to the ophidian pattern. On the evidence cited in this paper, and on a consideration of certain other cranial features, it is suggested that a close relationship between the Varanidae and the Serpentes cannot yet be regarded as firmly established.

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Abbreviations to Text-figures and Plate

<i>a ch</i>	anterior chamber of nasal sac	<i>ch</i>	choanal tube
<i>ad co</i>	aditus conchae	<i>ch g</i>	choanal groove
<i>add m</i>	adductor mandibulae muscle	<i>chor</i>	choroid
<i>a e n</i>	anterior external nostril	<i>c J o</i>	cartilage of Jacobson's organ
<i>a n t</i>	anterior nasal tube	<i>c l rec</i>	capsule of lateral recess
<i>aosp</i>	anterior space of nasal sac	<i>co</i>	nasal concha
<i>a sp</i>	anterior space of nasal sac	<i>co mu by</i>	concavity for mushroom body
<i>br</i>	brille (spectacle)	<i>con s</i>	conjunctival space
<i>cart</i>	nodules of cartilage beneath anterior cupola	<i>cor</i>	cornea
		<i>co z</i>	conchal zone of nasal sac

<i>cup</i>	anterior cupola	<i>old 1, old 2</i>	openings of lachrymal ducts 1 and 2
<i>d c z</i>	dorsal conchal zone	<i>ol ep</i>	olfactory epithelium
<i>d Hd gl</i>	duct of Harderian gland	<i>o s</i>	orbital sinus
<i>d J o</i>	duct of Jacobson's organ	<i>pal</i>	palatine
<i>d na gl</i>	duct of nasal gland	<i>pal a</i>	palatine artery
<i>d n p</i>	ductus nasopharyngeus	<i>p al in</i>	processus alaris inferior
<i>ec c</i>	ectchoanal cartilage	<i>p al su</i>	processus alaris superior
<i>ecpt</i>	ectopterygoid	<i>pas</i>	parasphenoid
<i>eth a</i>	ethmoidal artery	<i>p gl</i>	palatine gland
<i>eth g</i>	ethmoidal ganglion	<i>pmx</i>	premaxilla
<i>f</i>	flange of cartilage	<i>p mx a</i>	processus maxillaris anterior
<i>f end a</i>	fenestra endonarina anterior	<i>p mx p</i>	processus maxillaris posterior
<i>f endch</i>	fenestra endochoanal	<i>pmx b</i>	premaxillary bar
<i>f ex a</i>	fenestra exonarina anterior	<i>pn c</i>	paranasal cartilage
<i>f exch</i>	fenestra exochoanal	<i>pn c'</i>	separated part of <i>pn c</i>
<i>fl</i>	fenestra in nasal septum	<i>pn w</i>	postnasal wall (planum antorbitale)
<i>f olf</i>	fenestra olfactoria	<i>p pmx</i>	palatal process of <i>pmx</i>
<i>fo ap</i>	foramen apicale	<i>prfr</i>	prefrontal
<i>fo ep</i>	foramen epiphaniale	<i>ps c</i>	paraseptal cartilage
<i>fr</i>	frontal	<i>ps c'</i>	fragmented part of <i>ps c</i>
<i>f vo ex</i>	fenestra vomeronasalis externa	<i>pt</i>	pterygoid
<i>f vo in</i>	fenestra vomeronasalis interna	<i>pt c</i>	parietotectal cartilage
<i>gl</i>	gland	<i>r na c</i>	roof of nasal capsule
<i>Hd gl</i>	Harderian gland	<i>re ep</i>	respiratory epithelium
<i>hy c</i>	hypochoanal cartilage	<i>ros p</i>	rostral process
<i>i ch</i>	inner choana	<i>s b s</i>	sub brillar space (closed conjunctival space)
<i>invag</i>	invagination of wall of lachrymal duct 2 in <i>Varanus</i>	<i>smx</i>	septomaxilla
<i>i ob</i>	inferior oblique muscle	<i>so</i>	supraorbital
<i>i o s</i>	interorbital septum	<i>sph c</i>	sphenethmoid commissure
<i>J o</i>	Jacobson's organ	<i>st</i>	spongy tissue round anterior chamber of nasal sac
<i>ju</i>	jugal	<i>t</i>	tooth and dental lamina
<i>la</i>	lachrymal	<i>to</i>	tongue
<i>lc</i>	lachrymal canaliculus	<i>tr</i>	trabecula communis
<i>ld</i>	lachrymal duct	<i>v co, v o c</i>	vomerine concha
<i>ld 1, ld 2</i>	lachrymal ducts 1 and 2 in <i>Varanus</i>	<i>v c z</i>	ventral conchal zone of nasal sac
<i>lp 1, lp 2</i>	lachrymal puncta 1 and 2	<i>vo</i>	vomer
<i>l rec</i>	lateral recess of nasal sac	<i>vo cu</i>	vomerine cushion
<i>lta</i>	lamina transversalis anterior	<i>vo gl</i>	vomerine gland
<i>lta'</i>	free posterior end of <i>lta</i>	<i>vo pmx</i>	vomerine process of <i>pmx</i>
<i>mu by</i>	mushroom body	<i>x</i>	connexion between <i>ld 2</i> and cavity of nasal sac in <i>Varanus</i>
<i>mx</i>	maxilla	<i>I</i>	olfactory nerve
<i>mx'</i>	palatal process of <i>mx</i>	<i>I'</i>	vomeronasal nerve
<i>mx a</i>	maxillary artery	<i>Va</i>	ophthalmic and ethmoidal nerve
<i>na</i>	nasal	<i>Va m</i>	medial ethmoidal branch of <i>Va</i>
<i>na gl</i>	nasal gland	<i>Va l</i>	lateral ethmoidal branch of <i>Va</i>
<i>ns</i>	nasal septum	<i>Vb</i>	maxillary nerve
<i>o ch</i>	outer choana	<i>VIIp</i>	palatine branch of facial nerve
<i>od J o</i>	opening of duct of <i>J o</i>		
<i>ol b</i>	olfactory bulb		
<i>ol ch</i>	olfactory chamber of nasal sac		

EXPLANATION OF PLATE

A *Xenopeltis unicolor*, subadult Transverse section through right organ of Jacobson showing relationship of lachrymal duct to duct of Jacobson's organ ($c \times 260$) B *Tupinambis teguixin*, subadult Transverse section through left organ of Jacobson ($c \times 20$) C *Varanus monitor*, subadult Transverse section through right organ of Jacobson The relationship of the lachrymal duct to Jacobson's organ is similar in A, B and C ($c \times 36$) D *Varanus monitor*, subadult Transverse section through side of snout showing ending of lachrymal duct 1, and connexion (x) between lachrymal duct 2, and cavity of nasal sac ($c \times 36$)



A CYTOARCHITECTURAL INVESTIGATION INTO THE BOUNDARIES OF CORTICAL AREAS 13 AND 14 IN THE HUMAN BRAIN

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INTRODUCTION

Walker (1940) has described two new areas in the posterior orbital surface of the macaque brain—area 13 in the posterior orbital gyrus and area 14 in the gyrus rectus beneath and medial to the olfactory tract. Both areas were essentially agranular. No corresponding investigations have been reported in the human brain, nor did Walker fully discuss the relationship of his two new areas to previously described cortical subdivisions in man. Von Bonin (1944), however, tried to correlate almost agranular zones in the posterior orbital surface of the human brain, described by von Economo & Koskinas (1925), with an 'area orbitalis agranularis' homologous in all mammals so far investigated. As will become apparent, however, this correlation does not seem to cover entirely the more complex conditions which appear to obtain in man. In the light of these considerations, and since the neurophysiology of this region has lately aroused much interest, re-examination of its cytoarchitecture seemed to be a matter of some urgency.

Bailey & Sweet (1940) stimulated the gyrus orbitalis posterior in monkeys and cats causing inhibition of respiration, rise of blood pressure and decrease in the tonus of the gastric musculature. Ruch & Shenkin (1943) ablated area 13 in a series of monkeys producing a marked degree of hyperactivity. Fulton (1947) mentioned that experiments carried out jointly with Livingston confirmed the results of Ruch & Shenkin in monkeys as far as area 13 was concerned. Small bilateral lesions of area 13 in one chimpanzee, however, did not cause hypermotility, from which Fulton concluded that the region may be larger than in the monkey and in man may be larger still. Ward & McCulloch (1947) recorded neuronographic evidence of strong projections in monkeys from posterior and medial portions of the orbital surface (areas 10, 47, 53 in Bailey's terminology) to the paraventricular nucleus of the hypothalamus and from area 47 and posterior area 45 to the posterior hypothalamic area. More recently, Delgado & Livingston (1948) published their results obtained by electrical excitation on monkeys and dogs, which showed that the active region concerned with respiratory and blood pressure changes coincides with area 13 and that in some animals its limits are sharp. After bilateral ablation of area 13 their monkeys showed elevated temperatures in the hind limbs.

That this region in man seems to be concerned with similar functions, has been shown in a paper by Meyer & McLardy (1948), dealing with posterior cuts in prefrontal leucotomy, nine of their cases, with bilateral involvement of the central third

of the posterior half of the orbital region (liable to include the region corresponding to area 18 in monkeys), developed post-operative disturbances, many of which seemed to have an autonomic basis

Although the precise delineation of the agranular orbital cortex in man was the object of this study, it has been necessary, for the understanding of the correct boundaries, to include a cytoarchitectonic description of adjacent areas

MATERIAL AND METHODS

The cytoarchitectonic investigation of the orbital surface was carried out on nine hemispheres taken from seven different cases. Case 1, a normal male aged 40, was fully investigated in serial sections through the whole length of the left orbital region. Case 2, a normal male aged 20, was investigated in serial sections through the posterior half of the left orbital region, and through a sample block from the anterior half. Cases 3-7 were examined in serial sections through sample blocks from three different levels, chosen within the anterior, middle and posterior thirds of the orbital surface. This material (cases 3-7) was taken from four patients in whom prefrontal leucotomy had been performed without damaging the orbital region or its projections, and from one patient with an old arteriosclerotic softening around the Sylvian fissure.

The material, which was obtained approximately 24 hr after death, was fixed in 10 % formalin and subsequently embedded in celloidin. Serial sections (30μ) were cut, of which every twenty-fifth was stained by the Nissl method. In these comparatively thick sections all the peculiarities of each region were emphasized, without obscuring finer details. Some myelin-stained sections (Heidenhain's modification of Weigert's method) were also examined to exclude the presence of any small lesion not obvious in the Nissl preparations. No lesions were found.

Owing to the convolutional characteristics of the orbital region, i.e. arrangement of sulci and gyri more or less parallel to the medial and lateral surfaces (Text-fig 1*b*), only coronal sections were used. In this way the desired 'ideal plane' at right angles to the sulci and cortical surface was obtained and distortion of the cortical pattern due to obliquity of section was avoided.

The criteria employed for differentiation of the various areas were chosen with the greatest care, in order to avoid the criticism brought forward by Lashley & Clark (1946). Only striking alterations in the laminar pattern were regarded as a basis for subdivision and the characteristics of each area, as described below, could be recognized throughout the nine hemispheres. Minor differences of cell size, cell shape, or width of cortex, which could be interpreted as individual differences were noted, but never by themselves taken as an indication of a distinct cortical field. The above applies mainly to the criteria employed for parcellation in the medio-lateral axis, antero-posteriorly the subdivision was based on the presence or absence of an internal granular layer, a criterion which is valid beyond doubt.

It proved extremely difficult to carry out parcellation by microscopical observation only. Therefore, in case 1 photomicrographs of every hundredth section of the gyrus rectus, of the orbital gyri and of the pars orbitalis of the inferior frontal gyrus were taken and were fixed to a board in their correct topographical position. In this way a reasonably representative sample of the microscopic structure of the entire

orbital surface could be viewed simultaneously, an invaluable help for parcellation. In addition, the surface outline of these sections was drawn by projection and the boundaries of the architectonic areas marked in under microscopic control. The chart (Text-fig 1*a*), representing a reconstruction of the orbital surface of case 1, was based on these drawings. The level of each section has been shown by its corresponding serial number at the side of the chart, thus the exact location of photomicrographs (Pls 1–3) can be easily determined. Where wide transitional zones between two areas occurred they have been indicated, using the symbols of both areas concerned simultaneously. The next charts (Text-fig 2*a, b*) show the position of Brodmann's (1914, 1925) and von Economo & Koskinas' (1925) areas as they would appear, if projected on to the orbital surface of case 1. The thick lines in von Economo & Koskinas' chart indicate the location of their photographs.

Following Walker's example Brodmann's numerical nomenclature has been used as far as possible throughout the region investigated. It proved necessary, however, to introduce certain further terms: i.e. area recta anterior and posterior and area 47 anterior and posterior*. Reference to the most posterior extension of the orbital cortex—transition into insular and parolfactory regions—has been made in von Economo & Koskinas' terms *FJ* and *FL*, since, except for area 25, no terminology of Brodmann's is available. It seems likely that in future investigations von Economo & Koskinas' terminology will have to be introduced throughout as being more logical and more flexible.

RESULTS

The following areas have been recognized throughout the nine hemispheres, their position can be located from Text-fig 1*a*.

Area recta anterior and area recta posterior (Text-fig 1*a*)

Cytoarchitecture. Area recta anterior lies on the anterior half of gyrus rectus expanding laterally to cover the most medial part of the adjacent orbital gyrus. Anteriorly it terminates with the end of the olfactory sulcus, the subrostral sulcus forms its border on the medial surface.

Its main characteristics are: narrowness of the cortex as a whole, especially accentuated narrowness in layer III, cells of equal size throughout III, distinctly marked, though narrow internal granular layer and—in places—arrangement of the infragranular layers in vertical columns. None of the laminae is sufficiently well marked to give rise to horizontal striation (Pl 1, fig 1).

Area recta posterior covers the posterior half of gyrus rectus, and extends laterally into the most medial part of the adjacent orbital gyrus. Anteriorly it is bordered by area recta anterior, posteriorly it gradually merges into the parolfactory area of Broca (*FL* of von Economo & Koskinas). The sulcus subrostralis forms its boundary on the medial surface.

Area recta posterior may best be described as an agranular subdivision of area recta anterior, for it retains all its other characteristics (Pl 1, fig 2). The change from granular to agranular cortex is not a sudden one, but takes place over a fairly

* Brodmann already suggested a possible further subdivision of his area 11 into a medial area recta and a lateral area orbitalis interna on the strength of different cytoarchitecture. But he never actually executed this suggestion by drawing these areas separately on his chart.

extensive transitional zone in which the internal granular layer becomes gradually narrower, less dense and more intermingled with large cells from layers III and V. No difference in cortical structure could be noted between the crown of gyrus rectus and the walls of the olfactory sulcus.

Discussion The position and structure of area recta anterior and area recta posterior—as described in this paper—seem to be identical with von Economo & Koskinas' description of their areas *FG* (their plate XXXV) and the orbital portion of *FH*, respectively.

Brodmann's* area 11 includes in its most medial portion the site of our areas recta anterior and posterior. Its architecture, however, described as being uniform for the entire area, resembles only the structure in our transitional zone, between areas recta anterior and posterior.

A cytoarchitectonic parcellation of gyrus rectus was undertaken by Ngowyang (1932), who confirms on the whole Vogt's (1910) myeloarchitectonic subdivisions. Ngowyang distinguishes between anterior granular and posterior dysgranular portions, the former being roughly identical with area recta anterior of the present writer. Only a small area in the depths of the olfactory sulcus is described as quite agranular but is assigned to the parolfactory region.

Walker, in the macaque, did not describe any area comparable to our area recta anterior. His area 14, however, occupies a position apparently similar to our area recta posterior, but the description and illustration of its architecture—taken from the walls of the olfactory sulcus—show it to be much more akin to the parolfactory cortex (*FL*) in man, although the latter is restricted to about the posterior tenth of gyrus rectus. It therefore seems probable that his area 14 is not homologous with our area recta posterior, a view which finds further support from von Bonin & Bailey (1947) who, adopting von Economo's terminology for their map of *Macaca mulatta*, assign the entire area equivalent to Walker's 14 to parolfactory *FL* †.

Areas 11 and 13 (Text-fig 1a)

Cytoarchitecture Area 11 represents the cortex of the anterior two-thirds of the medial orbital gyrus, forming the lateral boundary of area recta anterior. It curves round the anterior end of the olfactory sulcus, extending slightly on to the medial aspect of the hemisphere. Laterally it encroaches upon the middle orbital gyrus.

Its cytoarchitecture is similar to area recta anterior, the main differences being gradual medio-lateral increase in total width, evenly distributed throughout the layers, a denser layer III with larger cells in its depths, which permit its subdivision into *IIIa* and *IIIb*, a more pronounced layer *Va* and layer *VIa* with lighter layer *Vb*,

* As the data on which Brodmann (1908, 1910, 1912, 1914 and 1925) based his cytoarchitectonic map of the human brain have never been published, Rose's (1935) detailed histological descriptions and photographs of Brodmann's areas have been used for the assessment of the architectonic characteristics.

† As far as can be seen from the literature no autonomic responses have so far been obtained from Walker's area 14 in animals. On the other hand, Fulton (1947) describes a human case in whom during the course of an operation for a pituitary tumour the most posterior portion of, what appears to be from his description, gyrus rectus was accidentally fixed bilaterally with Zenker's fluid. (This region would most likely include parolfactory as well as cortex from the posterior part of our area recta posterior.) The patient developed a condition similar to *sham rage* in animals.

giving rise to distinct horizontal striation, and no columnar arrangement of the infragranular layers (Pl 2, fig 1)

Area 13 covers the posterior third of the medial orbital gyrus, or the posterior fifth only, if the transitional zone be excluded. Anteriorly it is bordered by area 11, posteriorly by von Economo & Koskinas' area 'fronto-insularis' (*FF*) in the gyrus transversus insulae.

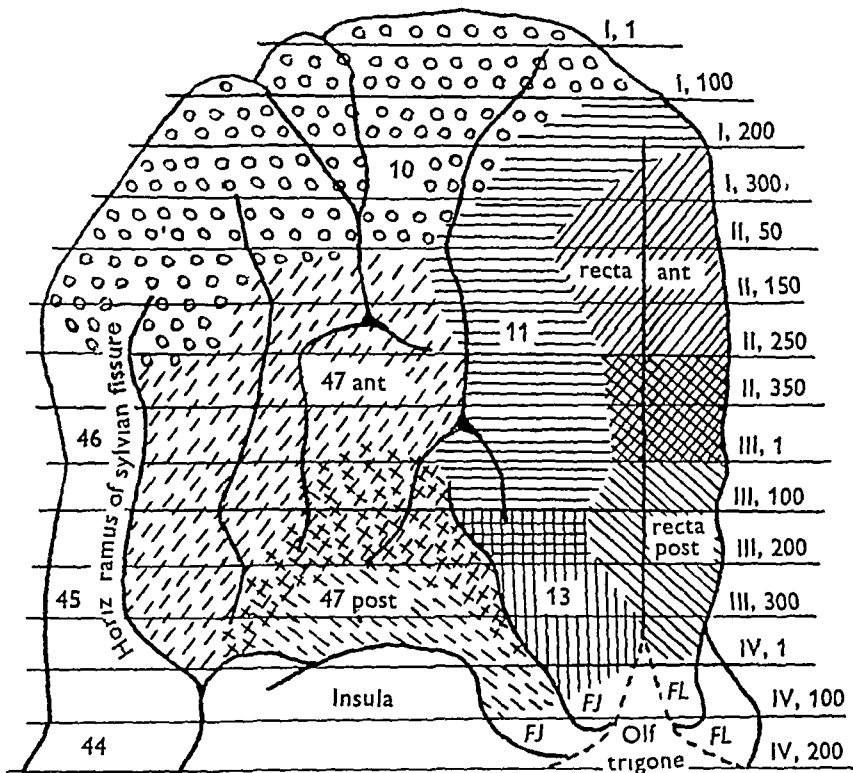
Just as I regard area recta posterior as an agranular subdivision of area recta anterior, so area 13 can be termed an agranular subdivision of area 11. It retains all the main characteristics of area 11, including a very marked layer *Va* with especially large cells towards the posterior end and a well-developed layer *VIa* (Pl 2, fig 2). The latter two features give rise to distinct horizontal striation, which also constitutes the main difference from area recta posterior. The transition from granular to agranular cortex is gradual, but extends only over 100 sections as compared with 200 on gyrus rectus.

Discussion. Neither Brodmann nor von Economo & Koskinas describe any area which is topographically or structurally identical with areas 11 and 13, as just defined. Topographically these areas fall within the lateral portion of Brodmann's area 11—i.e. his suggested area 'orbitalis interna'—for which, however, neither a separate structural description was given nor a further possible subdivision into granular and agranular parts considered.* With regard to von Economo & Koskinas' chart our areas 11 and 13 fall within the medial portion of their area *FF*. No part, however, of *FF* is described as similar to our 11 and 13, on the contrary representative photographs of the anterior granular and posterior agranular subdivisions (*FFg* and *FFa*), both taken from lateral portions, are identical in every detail with those of our areas 47 anterior and posterior, to be described later (Pl 3, figs 1 and 2). A comparison, however, of Pl 2, figs 1 and 2 with Pl 3, figs 1 and 2 will show the striking difference between our areas 11 and 13 on the one hand, and our 47 anterior and posterior on the other. Separation of areas 11 and 13 from the field *FF* seems therefore appropriate, and finds further support from Vogt's (1910) and Strasburger's (1937) description of the myeloarchitecture of this region, i.e. 'unistriar' corresponding to our areas 11 and 13, but 'bistriar' and 'unitostriar' to our areas 47 anterior and posterior, respectively.

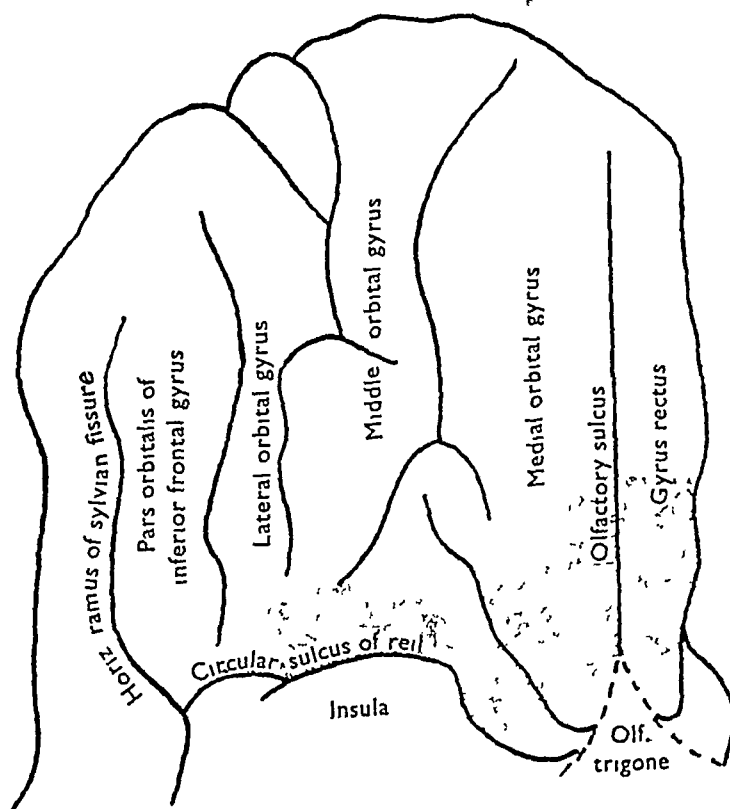
Walker's description and photographs of his areas 11 and 13 in the macaque show them to be homologous in topography and structure to those described in man in this paper. There exists only the difference in expanse, transition from areas 11 to 13 in the macaque occurring between the anterior and posterior half of the orbital gyrus, whilst in man it takes place between the middle and posterior third of the (medial) orbital gyrus. Thus area 13 in man is far more restricted.

In their charts of the macaque von Bonin (1944) and von Bonin & Bailey (1947) homologize Walker's area 13 with their areas 47 and *FF* respectively. It is not my intention to go into the often very contradictory evidence of homologies, in man, however, it must be stressed that investigation seems to show more complicated conditions, area 13 being by no means identical with area 47, even in the latter's most posterior agranular portion.

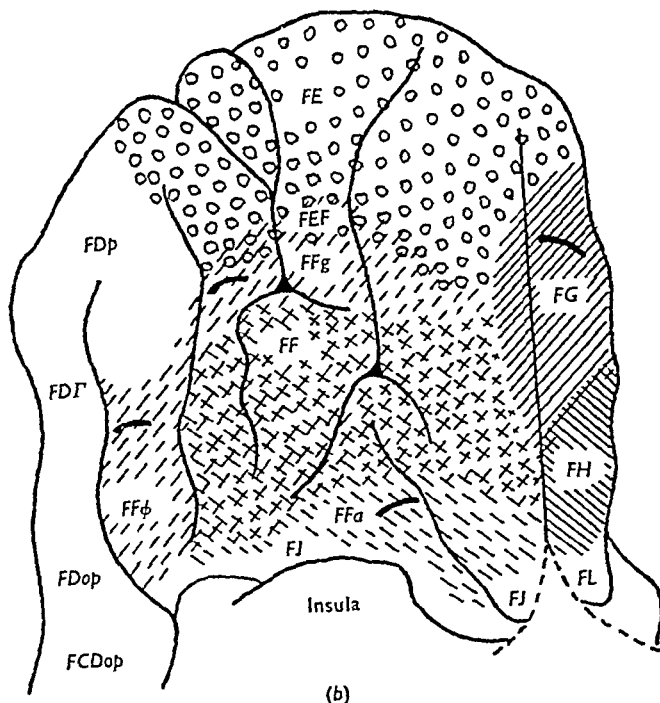
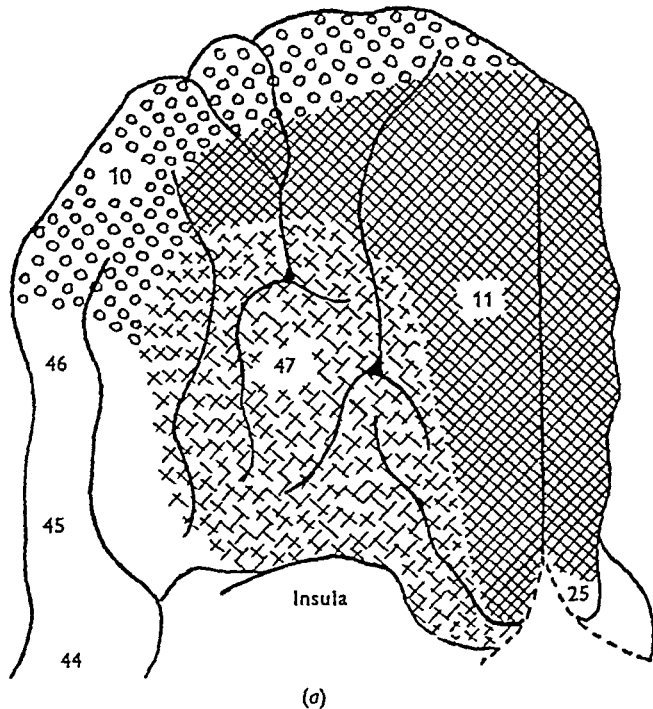
* Brodmann's original term 'area 11' has none the less been retained in this study on account of its customary usage in man and its homology to Walker's area 11 in the macaque.



Text fig 1a Parcellation of the orbital cortex in case 1, left, temporal pole removed. Reconstruction from diagrams of every hundredth section at a magnification of two (allowing 6 mm for hundred sections of 30μ thickness). The curvature of the brain has been flattened out.



Text fig 1b Diagram indicating the total amount of agranular orbital cortex (stippled area), and the terminology used for gyri and sulci.



Text fig 2 Brodmann's (a) and von Economo & Koskinas (b) areas projected upon the orbital surface of case 1. The markings have been adapted to those of case 1, i.e. equal markings signify identically described cytoarchitecture

Areas 47 anterior and posterior (Text-fig 1a)

Cytoarchitecture Area 47 anterior lies on the orbital portion of the inferior frontal convolution and extends medially to cover the lateral and middle orbital gyri. Anteriorly it is limited by the polar cortex (area 10), posteriorly by its agranular subdivision, area 47 posterior. The horizontal ramus of the Sylvian fissure provides its approximate lateral boundary, the medial orbital sulcus its medial one.

Area 47 anterior is differentiated from area 11, which it borders laterally, by a considerable increase in width and density of the cortex, especially marked in layers IV and VIa, the latter being far denser than in any of the previously described areas. The fifth layer also becomes wider but very light and cell-poor, especially Vb. This produces a very pronounced horizontal striation. There is also a tendency to arrangement of the cells in vertical columns (Pl 3, fig 1).

Area 47 posterior is a small zone which lies in the most posterior end of the lateral and middle orbital gyri. Anteriorly it is bordered by area 47 anterior, posteriorly by the anterior portion of the circular sulcus of Reil and von Economo & Koskinas' area fronto-insularis (FJ).

Area 47 posterior can best be regarded as an agranular subdivision of area 47 anterior whose main characteristics, i.e. emptiness of layer V and dense, well-developed layer VIa, are preserved. Horizontal striation, however, becomes indistinct owing to the lack of an internal granular layer, the arrangement in vertical columns becomes less marked, and the transition from fifth to sixth layer is in places difficult to assess (Pl 3, fig 2). In addition, the cortex becomes less dense and the individual cells larger as one progresses posteriorly. The increase in cell size applies mainly to layer Va, where indeed very large cells can be seen occasionally, nowhere, however, giving rise to distinct striation. This latter feature constitutes one of the main differences from area 13, which forms its medial boundary. The division of area 47 into granular and agranular cortex is less distinct than in previously described regions, the transitional zone—in some cases—being a much wider one, thus confining the completely agranular cortex to a narrow posterior strip in the lateral and middle orbital gyri. The cortex of the orbital portion of the inferior frontal convolution remains granular throughout its antero-posterior length.

Discussion There seems to exist considerable confusion in the literature with regard to the topography and structure of area 47, which may be attributable to its apparently great variability. Brodmann's area 47—forming part of the 'infrafrontal region' in his revised terminology—occupies a similar topographical position to our areas 47 anterior and posterior, but does not reach the horizontal ramus of the Sylvian fissure at any point (the granular cortex of this region being assigned to his areas 45 and 46). The description of its architecture, however, with a feebly developed internal granular layer and wide infragranular layers renders it akin only to our transitional zone between areas 47 anterior and posterior.

Von Economo & Koskinas would include the site of our areas 47 anterior and posterior in the lateral two-thirds of their area FF, giving, in contradistinction to Brodmann, the horizontal ramus of the Sylvian fissure as its lateral boundary. They stress the fact that this region has no uniform character and that it is therefore impossible to give a description of its architecture equally applicable to every part

They mention and illustrate besides an anterior granular portion, *FFg*, a posterior agranular *FFa* and a lateral granular *FF ϕ* . Apart from these they seem to have encountered considerable individual differences, *FF* showing in extreme cases a wide, dense internal granular layer throughout. Their representative photographs of *FFg* and *FFa* both taken from lateral portions (their plates xxxii and xxxiii), are, as already mentioned, in every detail identical with those of our areas 47 anterior and posterior, respectively. A region corresponding in structure to *FF ϕ* could also be clearly seen in the present material.

Kreht (1936*a*) studied the cytoarchitecture of the region equivalent to our areas 47 anterior and posterior, using Vogt's (1910) terminology. His results with regard to anterior 'granularity' and posterior 'dysgranularity' are essentially in accordance with the findings in this study.

Walker, in the macaque, describes no area 47, nor any area homologous to our areas 47 anterior and posterior. The orbital portion of his area 12, which occupies a similar position to our area 47, displays a very different cytoarchitecture, and his area 13 has been shown in the present investigation to be homologous to area 13 in man.

Von Bonin (1944) emphasizes the presence of a homologous area 47 ('orbitalis agranularis'), in all mammals so far investigated (including man), and identifies it with Walker's area 13 in the macaque. In his joint publication with Bailey (1947) he terms it *FF* and gives a description of its architecture very similar indeed to that of Walker's 13. In his publication on Galago (1945), however, he describes area 47 as displaying a light fifth and a dense sixth layer, indicating a certain structural relationship to our area 47 posterior. The question of homology must therefore remain open.

Some difficulties were encountered in delimiting area 47 anterior from area 10. For this reason no transitional zone has been drawn in on the chart (Text-fig 1*a*) and the boundary given should be regarded only as a rough indication.

Individual differences

The fact that the main characteristics of each region could be recognized in all nine hemispheres by no means excludes the presence of distinct individual differences. Width of cortex, cell size, structure and density and above all granularity varied greatly from case to case. It therefore seems possible that the discrepancies between the various findings of different observers may be largely due to their considering such individual differences too much as a basis for parcellation, as indeed has been pointed out by Lashley & Clark (1946). Whether individual differences also exist with regard to the extent of granular and agranular orbital cortex in antero-posterior direction, or with regard to the size of the architectonic areas as a whole—such as Kreht (1936*b*) actually demonstrated for the 'wider area of Broca'—cannot be established from the present material. This question will have to be investigated later in serial sections throughout the length of the orbital surface in a large number of brains.

CONCLUSIONS AND SUMMARY

The most striking fact brought out by this study is that in all the cases a granular area recta anterior and area 11 could be subdivided from an agranular area recta posterior and area 13. The difference between a granular and an agranular area 47 was not so distinct, because in some cases the transitional zone was wide and complete agranularity was reached only in the most posterior levels. The cortex on the orbital portion of the inferior frontal gyrus was granular throughout its entire length in all cases.

The new terms, area recta anterior and posterior and area 47 anterior and posterior, have been introduced to signify the division into granular and agranular cortex.

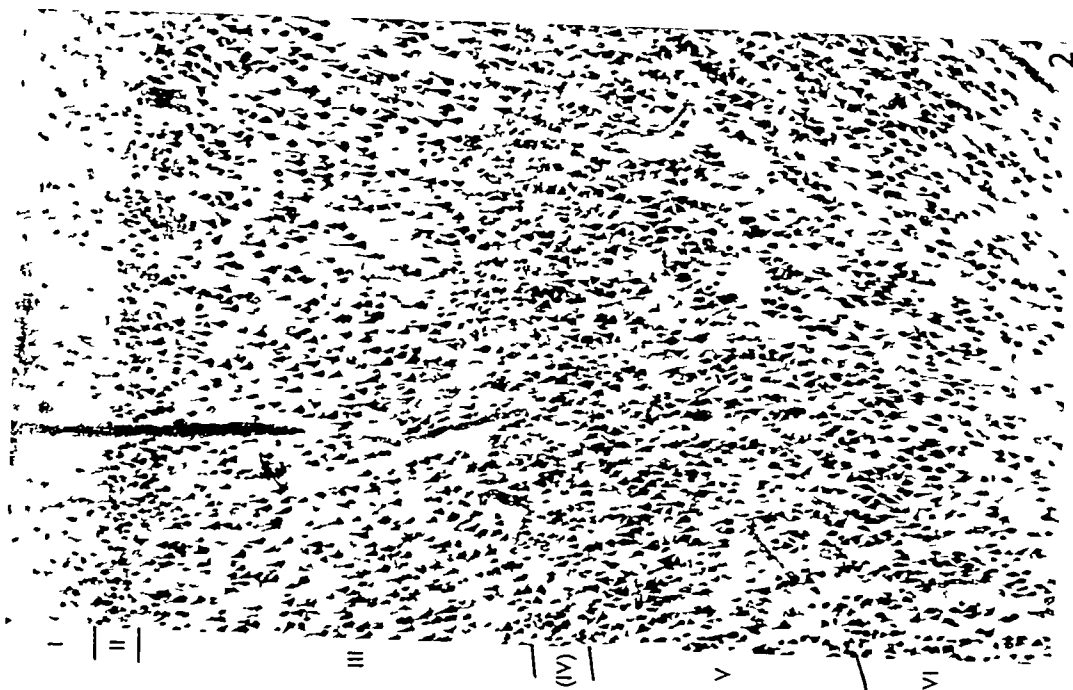
Homology of Walker's area 13 in the macaque to area 13 in man could be established, while homology of his area 14 in the macaque to area recta posterior in man seemed extremely doubtful. A different terminology, i.e. area recta posterior, was therefore chosen for the latter.

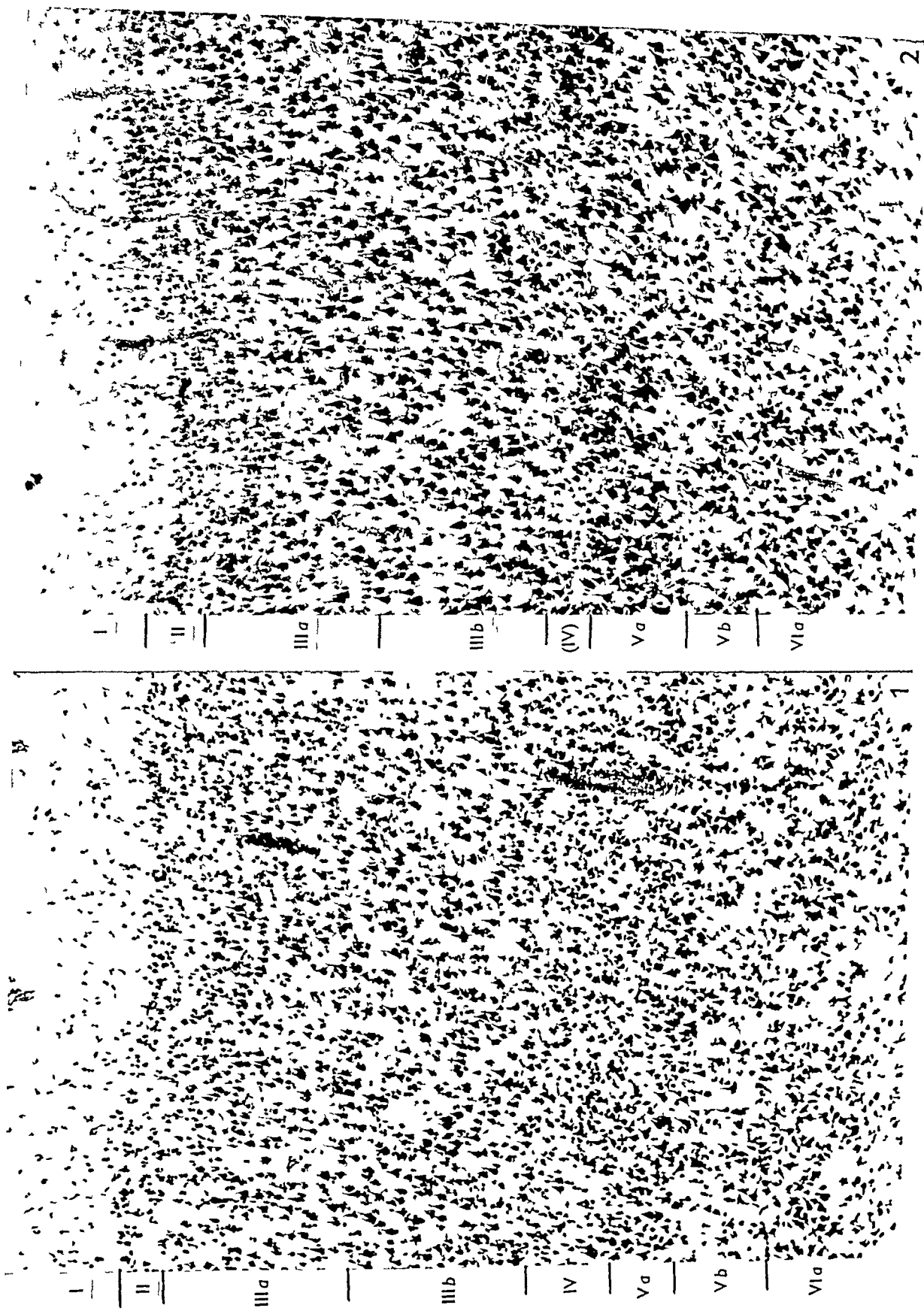
As compared with Walker's (1940) findings in the macaque the agranular portion of the orbital cortex in man seems to be proportionately much smaller. It is difficult to say where the exact anterior boundary should be drawn, as all these areas show a comparatively wide transitional zone with gradually decreasing internal granular layer in antero-posterior direction. This problem, however, might be solved if a more accurate functional boundary were determined. Meanwhile, it is interesting to note that this region belongs to the agranular type of cortex and is therefore more akin to premotor (area 6) than to prefrontal cortex.

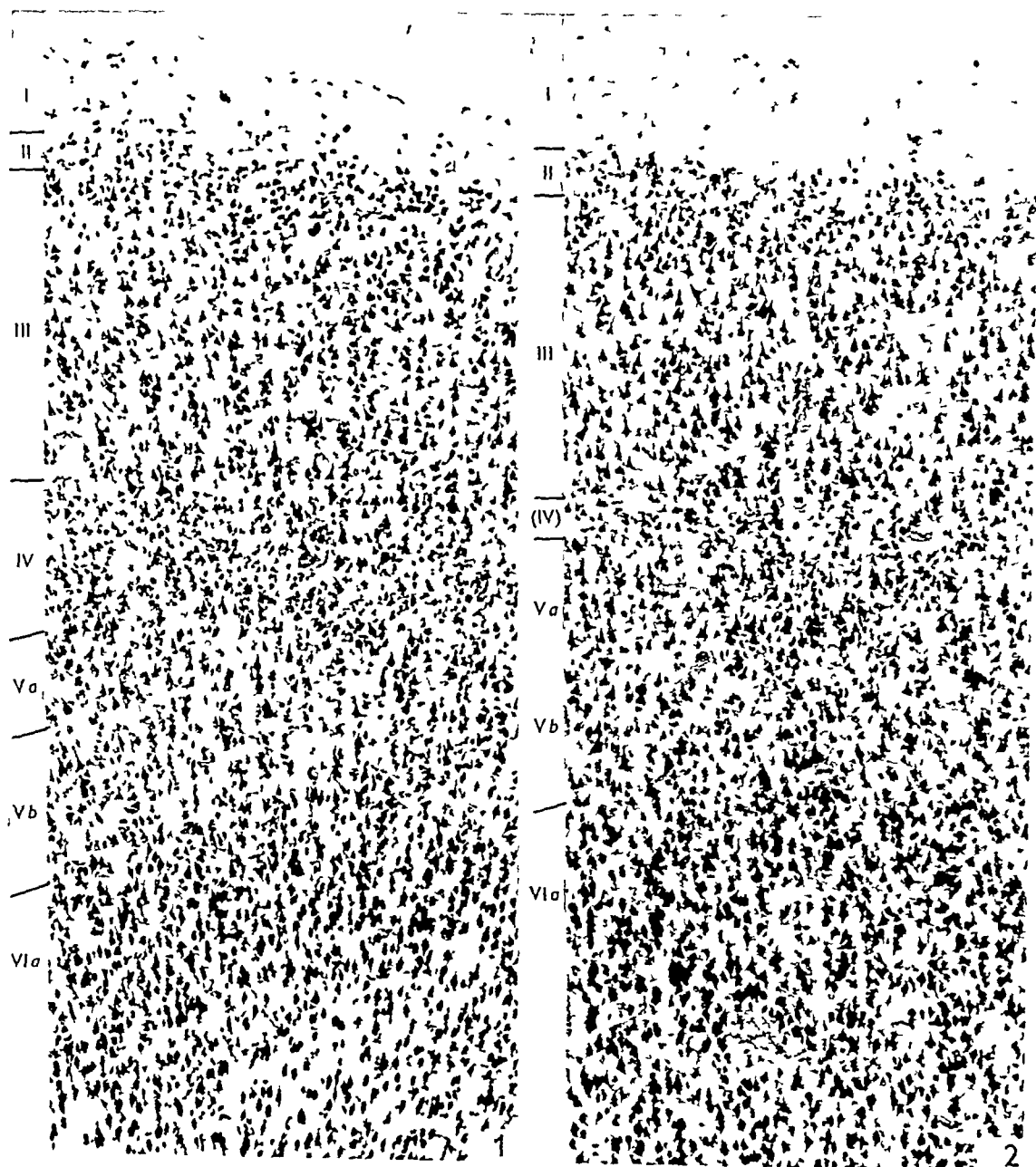
I wish to express my gratitude to Dr A. Meyer, at whose instigation this study was undertaken, for his criticism throughout.

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EXPLANATION OF PLATES

All the photomicrographs are taken from Nissl preparations at a magnification of $\times 85$. The '(IV)' in Pls 1, 2 and 3, fig 2, which illustrate agranular cortex, indicates the position of the outer stripe of Baillarger as seen in myelin stained sections

PLATE 1

- Fig 1 Case 1, left Bl II 150 Photomicrograph of area recta anterior, on the crown of gyrus rectus
- Fig 2 Case 1, left Bl III, 200 Photomicrograph of area recta posterior, on the crown of gyrus rectus

PLATE 2

- Fig 1 Case 1, left Bl II, 150 Photomicrograph of area 11 in the centre of the medial orbital gyrus
- Fig 2 Case 1, left Bl III 275 Photomicrograph of area 13, in the centre of the medial orbital gyrus

PLATE 3

- Fig 1 Case 1 left Bl II, 150 Photomicrograph of area 47 anterior, on the crown of the lateral orbital gyrus
- Fig 2 Case 1, left Bl III 275 Photomicrograph of area 47 posterior, in the centre of the middle orbital gyrus

BONES, MUSCLES AND VITAMIN C

I THE EFFECT OF A PARTIAL DEFICIENCY OF VITAMIN C
ON THE REPAIR OF BONE AND MUSCLE IN GUINEA-PIGS*

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INTRODUCTION

The observations described in this paper, some of which have been mentioned in earlier communications (Kodicek & Murray, 1943, Murray & Kodicek, 1946), were made in the course of an investigation, which had negative results, of the alleged reopening of old healed fractures in prolonged vitamin C insufficiency. We found no evidence of reopening of healed fractures under these conditions, but we made certain other observations, the first three of which are the subjects of the present paper. These observations concerned (1) the repair of the fractures, (2) the appearance of a contracture at the knee joints, (3) the failure of injured muscles to regenerate and their replacement by hyperplastic connective tissue, (4) the development of subperiosteal thickenings of the diaphyses of certain bones and other anatomical changes. These last are treated in the second and third papers of this series.

EXPERIMENTS, MATERIAL, METHODS

The animals used were guinea-pigs. The weights of the vast majority, at the beginning of the experiment, were between 200 and 400 g. The basal diet was in the earlier experiments 720 g oatmeal, 180 g bran, 20 g salt mixture, 100 g dried yeast, later, the bran and yeast were changed to 160 and 80 g respectively. To the basal diet we added ascorbic acid and radiostoleum. Carotene or carrots were given, as supplements of vitamin A, to the diets of those deprived of vitamin D. The supplements given in the different experiments are summarized in Table 1. In the diets which were partially deficient in vitamin C, our aim was to reduce the intake of the vitamin to as low a level as we could, while preventing obvious scurvy and death, in some experiments we gave 0.5 mg, in others 1 mg ascorbic acid daily. The animals were weighed on alternate days, or sometimes daily.

The fractures were inflicted under ether anaesthesia with forceps resembling those used by Hertz (1936). Following Hertz, the fibula was chosen for fracturing because the tibia made an effective splint and there was usually good apposition of the ends. No open operation was required, but the method undoubtedly involved injury to the soft tissues by squeezing.

* Parts II and III will appear in subsequent issues of the *Journal*.

† This work was done while the first author was at the Department of Biology, St Bartholomew's Hospital Medical College, London.

In all experiments, except Exp 2, a radiograph was taken to confirm that a fracture had been made, and further radiographs were taken later at intervals of from a fortnight to a month

The animals were killed with chloroform. The operated limb, and often both posterior limbs and other parts, were fixed in Heidenhain's 'Susa' solution. Specimens were at first embedded by Apathy's celloidin-paraffin method, but later in paraffin, the latter method provided more complete series of sections. Sections were stained with haematoxylin and eosin, Heidenhain's 'Azan', Wilder's silver method, van Gieson, Giemsa, and other methods. When animals died before being killed, similar material was collected and preserved in either 10 % formol or Susa.

Table 1 summarizes the nine experiments, showing how the diets were varied, the numbers of animals, the dates of the operation when one was performed, and the dates of death. 'Experiment 1' is really a list of all the dietetically normal animals, including some of those also listed as group 1 in Exps 2-6, in which a fractured fibula was studied histologically.

PARTIAL DEFICIENCY OF VITAMIN C AND DEPRIVATION OF VITAMIN D, AND THE REPAIR OF FRACTURES

The diets deficient in vitamin D, in which the balance of salts was not altered, produced neither rickets nor any delay in callus formation at the fractures, nor indeed any other effect, whether alone or combined with a partial deficiency of vitamin C.

The effects of partial and complete vitamin C deficiencies on early stages in the repair of bone have been studied by Bourne (1942, 1943, 1944) and others (Hanke, 1935, Hertz, 1936, Israel, 1926, Israel & Frankel, 1926, Jeney & Korpassy, 1934, Lexer, 1939, Roegholt, 1931-2, Schilowzew, 1928, Watanabe, 1924, Wolbach & Howe, 1925, 1926), but the influence of prolonged partial deficiencies, which we studied in Exps 2-6 is less well known.

The radiographic findings and the results of the microscopical investigation will be discussed separately.

Radiographs

In Exp 5, radiographs were taken of the operated limbs immediately after the operation and at 17, 32, 59, and 94 days later, and in Exp 6 immediately after the fracture and at 19, 50 and about 80 days after the fracture. In group 1 (fully supplemented diets) of both experiments, the radiographs (Pl 1, figs 1, 3) taken at 17 or 19 days showed a callus with a narrow uncalcified line across it, while in those taken at 32 or 50 days no such line was visible and the callus was so reduced as to make the exact position of the fracture difficult to recognize. In groups 3 (partially deficient in vitamin C) and 4 (partially deficient in vitamin C and deprived of vitamin D), the amount of callus formed (at least at the site of the fracture) at 17 or 19 days was much less (Pl 1, figs 2, 4). In addition, the resorption of bone from the broken ends was usually greater than in the control animals (group 1) as shown by the greater width of the clear line between them. Even at 32 or 50 days, traces of the gap, or even a clear area right across the bone, were often visible (Pl 1, fig 2).

TABLE 1 *A summary of the dietary vitamin supplements given, of the times at which supplements were changed, of the times of operation, and of dates of death Exp 1 includes all animals, on fully supplemented diets throughout, whose fractured fibulae were sectioned*

Exp 1	Group —	No of animals 34	Days of experiment —	Vitamin C supplement	Vitamin D supplement Fully supplemented	Vitamin A supplement	Day of operation	Days of experiment on which animals died or were killed (in brackets number of animals)
2	1	10	1-14 15-25 26-end	Cabbage None 0.5 mg A A daily*	6 drops radiostoleum weekly		1	1-7 (1 each), 9 (2), 10-14 (1 each), 15 (4) 16 (5), 63 (1), 81 (2), 83 (2), 84 (2), 85 (1), 94 (2), 101 (1)
	2	4	1-end	Cabbage	"	"	1	25 (1), 30 (1), 33 (1), 42 (1), 53 (1) 60 (1), 65 (1), 85 (3)
3	1	7	1-end	10 mg A A daily	6 drops radiostoleum weekly		1	85 (4)
	2	8	1-end	10 mg A A daily	6 drops radiostoleum weekly		1	16 (3), 102 (4)
	3	4	1-15 16-32 33-60 61-end	10 mg A A daily 0.5 mg A A daily 1.0 mg A A daily 0.5 mg A A daily	None 8 g carrots weekly 6 drops radiostoleum weekly		1	16 (3), 18 (1), 54 (1), 102 (3) 42 (1), 102 (3)
	4	12	1-15 16-32 33-60 61-end	10 mg A A daily 0.5 mg A A daily 1.0 mg A A daily 0.5 mg A A daily	None 8 g carrots weekly " " " " " "		1	31 (1), 32 (1), 42 (1), 69 (1), 88 (1) 102 (2), 103 (5)
4	2	4	1-end	10 mg A A daily	None	8 g carrots weekly	1	15 (1), 81 (3)
	4	1	1-14 15-37 38-end	10 mg A A daily 1.0 mg A A daily 0.5 mg A A daily	" " " " " "		1	81 (4)
5	1	4	1-end	10 mg A A daily	6 drops radiostoleum weekly		27	121 (4)
	2	4	1-end	10 mg A A daily	None	10 drops carotene twice weekly	27	52 (1), 91 (1), 107 (1), 121 (1)
	3	5	1-7 8-18 19-end	1.0 mg A A daily None 0.5 mg A A daily	6 drops radiostoleum weekly		27	63 (1), 67 (1), 113 (2), 121 (1)
	4	5	1-7 8-18 19-end	1 mg A A daily None 0.5 mg A A daily	None " " " "	10 drops carotene twice weekly	27	45 (1), 47 (1), 63 (1), 93 (1), 120 (1)

Exp	Group	No of animals	Days of experiment	Vitamin C supplement	Vitamin D supplement	Vitamin A supplement	Day of operation	Days of experiment on which animals died or were killed (in brackets numbers of animals)
6	5	2	1-7 8-18	1 mg A A daily None	6 drops radiostoleum weekly	6 drops radiostoleum weekly	None	62 (1), 121 (1)
	6	2	19-end	0.5 mg A A daily	10 drops carotene twice weekly	10 drops carotene twice weekly	None	61 (1), 63 (1)
			1-7	1.0 mg A A daily	"	"		
			8-18	None	"	"		
			19-end	0.5 mg A A daily	"	"		
	1	4	1-end	10 mg A A daily	6 drops radiostoleum weekly	6 drops radiostoleum weekly	24	98 (1), 105 (2), 109 (1)
	2	4	1-end	10 mg A A daily	10 drops carotene twice weekly	10 drops carotene twice weekly	24	47 (1), 79 (1), 85 (1), 99 (1)
	3	4	1-7	1.0 mg A A daily	6 drops radiostoleum weekly	6 drops radiostoleum weekly	24	40 (1), 51 (1), 95 (1), 109 (1)
	4	5	8-18	None	"	"		
			17-end	0.5 mg A A daily	"	"		
			1-7	1.0 mg A A daily	10 drops carotene twice weekly	10 drops carotene twice weekly	24	30 (2), 51 (1), 65 (1), 85 (1)
			8-18	None	"	"		
7	5	2	17-end	0.5 mg A A daily	6 drops radiostoleum weekly	6 drops radiostoleum weekly	None	95 (1), 109 (1)
			1-7	1.0 mg A A daily	"	"		
			8-18	None	"	"		
			17-end	0.5 mg A A daily	"	"		
	6	2	1-7	1.0 mg A A daily	10 drops carotene twice weekly	10 drops carotene twice weekly	None	72 (1), 109 (1)
			8-18	None	"	"		
			17-end	0.5 mg A A daily	"	"		
			1-10	None	6 drops radiostoleum weekly	6 drops radiostoleum weekly	None	54 (1), 62 (1), 64 (2), 66 (8)
	2	8	11-end	0.5 mg A A daily	"	"		
			1-10	None	6 drops radiostoleum weekly	6 drops radiostoleum weekly	None	72 (1), 86 (1), 107 (6)
			11-66	0.5 mg A A daily	"	"		
			67-end	10 mg A A + cabbage daily	"	"		
9	1	6	1-end	None	6 drops radiostoleum weekly	6 drops radiostoleum weekly	None	16 (1), 17 (5)
	2	8	1-16	None	6 drops radiostoleum weekly	6 drops radiostoleum weekly	None	66 (8)
			17-end	15 g cabbage daily	"	"	None	
	—	16	1-8 9-18 19-end	1.0 mg A A daily None 0.5 mg A A daily	6 drops radiostoleum weekly	6 drops radiostoleum weekly	27	29 (3), 31 (3), 34 (3), 37 (2), 40 (2), 42 (1), 44 (2)

* A A = Ascorbic acid

Microscopical investigation

Sections confirmed these differences between the dietetically normal and partially vitamin C-deficient animals

Normal animals

It is not necessary to give a full description of normal fracture repair, but certain points must be mentioned for comparison with events in the animals on experimental diets. The formation of new bone did not begin at the fracture itself, but at some distance from it in the swollen cambial layer of the periosteum, later, it gradually approached the fracture site. At about the end of the first week after the operation, cartilage might begin to appear in the densely cellular tissue in which the broken ends were now embedded. One of the two animals (Pl 2, fig 5) killed at 9 days showed a considerable quantity of cartilage forming in the fracture region, while the subperiosteal bone had reached the fracture and trabeculae growing out from the proximal and distal fragments, each towards the other, tended to link up around it. In the other 9-day specimen (Pl 2, fig 6) killed at this time, neither bone nor cartilage had yet appeared at the fracture site, though subperiosteal bone was actively developing at a short distance from the broken ends. In all the animals killed at from 10 to 14 days (Pl 2, fig 7), bone formation had spread to the fracture, and trabeculae had formed across it, making a sort of basket of trabeculae connecting the proximal and distal subperiosteal tissues. At first no bone formed centrally, between the broken ends themselves, but centrally situated trabeculae were present after 14 days. Cartilage was present in most cases. It tended to form in the peripheral parts of the cellular tissue which joined the fragments before the appearance of bone, but it was later often found in the central region between the ends. It was finally eroded and replaced by bone. In succeeding days (Pl 2, fig 8) the trabeculae of the callus became stronger and more numerous, and the cartilage, except for vestiges enclosed in bony trabeculae, disappeared.

The condition of the fractured region was investigated in eleven animals killed at times ranging from 81 to 101 days after the operation (Pl 3, fig 9). In all these the callus was recognizable in longitudinal sections from its disorderly structure. It differed from the callus of 2-3 weeks after fracture in two important respects. First, further deposition of bone on the trabeculae, and doubtless the formation of new trabeculae, had transformed the lightly built early callus into massive bone in which the solid substance predominated over the vessel-containing spaces, the opposite of the earlier condition. Secondly, the marked local expansion of the fibula, made by the early callus, had disappeared, and sections showed that the projecting part had been removed by osteoclastic resorption from the surface.

Partially vitamin C-deficient animals

Fracture repair in animals placed on the experimental diets before the infliction of the fracture was studied in Exps 5, 6 and 9. The material consisted of longitudinal sections through the fractured region in animals killed at the following number of days after operation (the numbers in brackets give the numbers of days between

beginning the experimental diets and death) 14 (40), 16 (42), 18 (45), 20 (47), 27 (51), 36 (63, two animals), 40 (67), 61 (85), 86 (113), 93 (120)

The tendency, seen in the radiographs, of many animals on the experimental diets to show more resorption at the broken ends than the controls did, was confirmed by the animal killed at 14 days (Pl 3, fig 10) in which there was a long gap between the fragments, filled by a cellular and fibrous tissue. No repair had yet begun at the fracture itself. In none of the animals on the normal diet and killed after 9 days was the repair process still in so early a stage, in all, cartilage had formed and bony trabeculae were crossing the gap.

In the experimental animals which died or were killed at 16, 18 (Pl 3, fig 11) and 20 days after the operation, a few trabeculae had joined across the gap, making a feeble connexion, but there was no cartilage, and the repair accomplished was very much less than in the normal animals. There was, however, great variation in the effect of the diet, and the animal which died at 18 days showed repair indistinguishable from that expected in an animal on a normal diet.

In most of the material from animals kept for longer periods, the new subperiosteal bone which, as in normal animals, first developed at some distance from the site of the fracture, had increased enormously and had formed a great thickening often extending along the whole length of the diaphysis. These diaphyseal thickenings, which will be described in detail in the second paper of this series, were trabecular structures resembling lightly built calluses of great extent. In the two animals at 36 days after the operation, and in that at 61 (Pl 3, fig 12), the two pieces of the fibula were connected by the new bone extending over them like an immense callus. In the one which died at 93 days a pad of cartilage remained, still unresorbed, between the ends (Pl 3, fig 13). In those at 40 and 86 days, in which no large thickening had developed, they were weakly joined by small calluses developed between and around their ends. The chief fact of interest is that, whether the region of the fracture was, or was not, enclosed in a large mass of new bone, the repair of the fracture was never completed as it was in the controls, for the callus, large or small, always remained of light trabecular construction and was never consolidated as it was in the animals on properly supplemented diets. Compare Pl 3, figs 12 and 13, with fig 9.

Pl 4, fig 14, shows the fractured fibula of an animal of Exp 4, group 4, in which the partially deficient diet did not begin until 2 weeks after the operation, and in which there was no period of total deficiency. Nevertheless, the callus, which must have been formed when the partial deficiency began, failed to consolidate. At least one other case behaved similarly.

STIFFNESS OF THE KNEES

The most constantly observed change at the joints was a development of stiffness at the knees. This occurred in the great majority of partially vitamin C-deficient animals. Thus, in Exp 9, in which the condition was most completely recorded, some degree of stiffness was found in both legs of all sixteen animals. It was never seen in guinea-pigs adequately supplied with vitamin C, and we did not notice it in other joints. Usually the leg was fixed in flexion, but sometimes in extension, in

severe cases we were unable to move the joint without using force which we feared would cause a fracture, while in others the knee could be moved with less violence but with evident pain. The condition has been described by Meyer & McCormick (1928), who give a photograph. Stiffness did not occur in animals provided with vitamin C but deprived of vitamin D.

The stiffening of the knees seems to be one of the earliest signs of a partial deficiency of the vitamin. In Exps 5, 6 and 9 it was already present on the day of the operation, that is on the 24th and 27th days of the experimental diet, which had included a period of total deficiency of vitamin C followed by partial deficiency. Unfortunately, we do not know whether, in the present experiments, the stiffness appeared during the period of total deficiency or not, but in more recent experiments by one of us (E K) it did not appear during total deprivation lasting 18 days. That total deficiency is not necessary for the appearance of stiffness is shown by Exps 3 and 4, in which there was no total deprivation, and in which stiffness appeared in at least thirteen out of thirty-two legs examined.

Stiffness is also not a result of the operation, for in Exps 3-6, among thirty legs operated, stiffness developed in nineteen but not in the other eleven, while of twenty-six unoperated legs, seventeen showed stiffness.

There was a distinct correlation between the right and left sides of the same animal in the experiments just mentioned, fifteen showed stiffness of both legs, eight of neither, and only three showed it in one leg and not in the other. Stiffness thus depends rather on the diet than on the injury.

Meyer & McCormick (1928) attributed stiffness to changes which they observed in the spinal cord. We made no study of the nervous system, but found that the knees remained stiff after death, and in Exp 9 we studied the effect of removing the muscle crossing the knee joint. This lessened the stiffness in ten cases but left it unaffected in nine. We are thus disinclined to regard the condition as wholly of central nervous origin. In the next section we shall describe a swelling of the soft tissues of the shank, with degeneration of the muscles and their replacement by a mass of hyperplastic connective tissue. This condition would certainly help to prevent active movement of the limbs and in extreme cases the connective tissue, by packing around the joint and thickening its capsule, may have lessened freedom of movement. The articular surfaces themselves nearly always appeared normal. An attempt to correlate stiffness of the knees with pathological changes in the musculature has an indefinite result. In the animals more severely affected by the partial deficiency, there were such characteristic scorbutic changes as haemorrhages about the knee and even into the joint cavity, and this doubtless contributed to the stiffness.

We incline to the view that stiffness is caused locally rather than in the central nervous system, and that a number of histological changes contribute, these including degenerative and oedematous changes in the muscles, and the formation of hyperplastic connective tissue at and around the joint.

MUSCLE DEGENERATION AND CONNECTIVE TISSUE HYPERPLASIA

When guinea-pigs in which a fibula had been fractured and which had been kept for long periods (up to 4 months) on diets deficient in vitamin C were killed, it was frequently noticed that one or both posterior limbs were swollen in the shank region, with tightly drawn skin, a change which did not occur in dietetically normal controls. On dissection, the flesh had a curious gelatinous feel and it was difficult to separate the muscles from one another, often they seemed to have been replaced by a tissue of a different character.

Structure of the altered tissue

The altered tissue was studied in Exps 3-6. Transverse sections of the swollen shank regions of animals from the partially vitamin C-deficient groups showed that there had been a great increase in the amount of loose connective tissue which, in severe cases, had largely replaced the muscles (Pl 4, figs 15, 16). In animals kept for such long periods as 3 months after the operation this tissue had the following character. The cells present were almost all of one kind, and resembled fibroblasts. The intercellular tissue components were a fibrillar framework and the tissue fluid. The fibrillar framework varied a good deal in character. The fibrils were extremely fine, and were often so knitted or matted together as to form the membranous walls of a honeycomb system, in the multitudinous, and of course communicating, cavities of which was the tissue fluid (Pl 5, fig 23). The picture was reminiscent of an argyrophil reticulum rather than of a mature areolar connective tissue with its interlacing collagen fibres as its dominant feature. The fibrils stained black with Wilder's silver method, except for the very finest, which we did not succeed in staining at all but made visible by mounting in 'Euparal'. The fibrils took only a weak blue colour with Azan and did not stain with van Gieson. As well as this architecture of extremely fine fibrils, collagen fibres were often also present, and might be a prominent feature in the histological picture.

Besides the formation of the hyperplastic connective tissue replacing the muscles, the normal connective tissue structures, such as fascia and the fibrous layers of periosteum, tended to break down. Fasciae might become completely unrecognizable. The fibrous layers of periosteum never broke down completely, but areas could be found in which the limiting fibrous membrane between the osteogenic tissue and the surrounding hyperplastic connective tissue had suffered dissolution, leaving no histologically recognizable barrier between them (Pl 4, fig 17). A similar fate might befall the interosseous ligament between the tibia and fibula, and even tendons.

When muscles were present in the section, depending in part on whether or not they were removed at dissection, they might be normal. On the other hand, they were often oedematous, the fibres not being packed closely together as in a normal muscle, but having wide spaces between them. The connective tissue framework of the muscle might be increased in quantity, as though the muscle were being invaded by the extra-muscular connective tissue, or its own connective tissue proliferating. The muscle fibres had often shrunk away from their envelopes and had lost their characteristic internal structure, the myofibrils being fused into a homogeneous mass.

In places, groups of more or less isolated muscle fibres might be seen wandering

through the hyperplastic connective tissue by which they were surrounded and separated from one another. Such muscle fibres usually seemed to have retained their structure, showing nuclei and striations, but were very small, often extremely so. We find it very difficult to decide whether they were fibres in process of destruction by atrophy, or regenerating fibres which had failed to grow to anything like their normal final size.

Vascularization of the hyperplastic connective tissue was always poor, vessels were few and often without blood corpuscles, especially the arteries. Arteries were frequently seen to have their lumina partially or completely blocked by thickening and vacuolation of the endothelium (see below). Often, but by no means always, large numbers of red blood corpuscles could be seen lying in the tissue spaces of large areas of the connective tissue.

Development of hyperplastic connective tissue

The origin and development of this tissue was studied in Exp. 9, in which sixteen young guinea-pigs on a partially vitamin C-deficient diet were killed after fracture of the fibula at intervals given in Table 1. Transverse serial sections were cut across the operated limbs, above and below the site of fracture, and a few longitudinal series were also made.

There was little or no hyperplasia of the connective tissue in animals killed at 3 days after the fracture, but its formation had begun at 5 days, was more obvious at 8 days, and was large in the animals killed at 11 days and later.

The formation of the new tissue was closely bound up with degenerative changes in the muscle. These were seen in the animals killed at 3, 5 and 8 days after the operation, and in one killed at 11 days. In animals killed later, muscular degeneration was much less apparent, but much muscle had disappeared. These changes resulted in the total destruction, so far as could be seen in transverse sections, of many or all of the muscle fibres in entire muscles. The earliest recognizable departure from the normal was an oedema of the muscles in which the fascicles, and also individual muscle fibres, became widely separated from one another (Pl. 5, fig. 18). So far, the individual muscle fibres appeared normal. This simple oedema was seen especially in the earlier killed animals, but was also to be seen much later, it could be, but was not necessarily, a prelude to the destructive changes next to be described. These began with a loss of nuclei and a disappearance of the fibrillar structure of the muscle fibres (Pl. 5, fig. 19). It was difficult to determine exactly what happened to the nuclei, they were either present and apparently normal, or absent. They did not become pycnotic, for such a change would have been easily recognizable, and we think some of them disappeared by a progressive loss of their staining power. In less injured muscle fibres, the nuclei survived and might later take part in what little regeneration occurred. The loss of fibrillar structure appeared as a fusion of the fibrils, making the fibres appear homogeneous in cross-section. This change was often accompanied by an increase in the diameter of the fibre and by a development of vacuoles, these appeared first as many tiny droplets, giving the cross-section of the fibre a pitted appearance (Pl. 5, fig. 20). Later, the droplets might coalesce and form a large central cavity by which the fibre might be blown out to a very large size. Vacuolation of the fibres was

common but not universal, a great many fibres acquired a 'fluffy' appearance and broke up into irregular fragments. Even in muscles in which most of the fibres were undergoing these changes, a few fibres here and there often seemed to remain normal.

While muscle fibres were degenerating, the muscles were invaded by cells from without. Most of these were rather large cells with much cytoplasm and oval or indented nuclei, of the type of mononuclear wandering cells. Mitotic figures were common among them. They were found in large compact masses, having a pseudo-epithelial appearance, in the peripheral parts of the sections. In life, these groups must have been close beneath the skin. From the masses, it was easy to trace migration of the cells to the muscles, into which they penetrated. Within the muscles, they applied themselves to the surfaces of the degenerating muscle fibres, and gave every appearance of being engaged in their destruction (Pl 5, fig 21). Multinucleate giant cells were also present, and similarly appeared to be engaged in attacking the degenerate fibres. At a somewhat later stage the muscle fibres, or many of them, had disappeared, while their endomysial or sarcolemmal envelopes persisted as empty tubes whose walls no longer gave with Azan the blue reaction so brilliantly seen in neighbouring normal muscles. The wandering cells could often be seen in occupation of these tubes in place of the original owners (Pl 5, fig 22).

While the destruction of muscle fibres was in progress, there was an increase in the fibroblasts between them. This was brought about partly by multiplication of cells in the original connective tissue of the muscle and partly, we suppose, by transformation of the wandering cells. Up to 8 days after the operation, degenerating muscles infiltrated by wandering cells are a predominant feature in the histological picture, while increase in the connective tissue is not yet striking, but from 11 days onwards such gross degenerative changes are scarce or absent while the striking histological feature is the great masses of young connective tissue cells occupying areas which had evidently been muscular, and in which traces of the envelopes of muscle fibres could often easily be seen. In such regions, the fibroblasts of the new connective tissue seem at first comparatively scarce, but there are mitotic figures among them. They multiply, and as they do so a delicate fibrillar reticulum forms, replacing the old architecture of the destroyed muscle, and this soon becomes unrecognizable.

At the same time, fibroblasts of the intermuscular connective tissues also proliferate and so contribute to the developing mass of new connective tissue.

A second mode of formation of the connective tissue was by proliferation of the fibroblasts between the muscle fibres of oedematous muscles whose fibres did not degenerate as described above but, we believe, were reduced in size and number by an atrophy which ended in the disappearance of many fibres.

Still a third mode of origin of the new tissue was by the organization of exudates, which were commonly found in the intermuscular connective tissue. By the penetration of fibroblasts into such exudates, they were built up into parts of the general connective tissue.

It was impossible to tell how much of the hyperplastic connective tissue formed by each of these modes of origin, because the final result was in each case the same, but the largest contribution was certainly made by the destruction of muscle fibres and their replacement by connective tissue.

The connective tissue, formed as just described, did not, within the limits of the

experiment (extending up to 18 days after the fracture), become identical in structure with the fully formed hyperplastic connective tissue which we have described in material fixed some 3 months after the operation. Large parts of it are much more densely cellular than it later becomes, and the honeycomb-like architecture, seen in the older material, has not yet developed, the fibrillar framework making a much more open net-work.

Regeneration of muscle fibres

In the developing hyperplastic connective tissue one could very often see a number of very small muscle fibres, wandering in an apparently undirected manner. Many of these were undoubtedly regenerating muscle fibres, for they showed multi-nucleate regeneration buds, but we suspect that some were fibres in course of atrophy. The regenerates, even in animals which lived for 3 months or more after the operation, remained very much smaller than normal fibres and were few in number, nothing suggested that any more complete regeneration of muscle fibres occurred, such as was described by Le Gros Clark & Blomfield (1945), and as we found in our dietetically normal animals. If such extensive regeneration had occurred in the dietetically partially deficient animals, the observations described above, of the replacement of large areas of muscle by connective tissue, could not have been made.

Comparison with dietetically normal animals

The changes described above in animals on diets partially deficient in vitamin C were also seen in those on fully supplemented diets (Exp 1, and group 1 of Exps 3-6), but there was a great quantitative difference.

In the three dietetically normal animals killed on the 1st, 2nd and 3rd days after the fracture, a considerable number of changed muscle fibres were found. These fibres showed loss of nuclei, loss of striations, and loss of myofibrils, giving the fibres a homogeneous appearance. Some fibres suffered fragmentation. The muscles were oedematous and there was an extensive polymorph infiltration. Whether there was markedly less of these changes in the first 3 days than was to be seen in the two partially deficient animals killed at 3 days, it is difficult to say.

In the animal killed at 4 days after the fracture, the polymorph infiltration was much less, there was more than the normal amount of connective tissue and this was very cellular and obviously recently formed. Since regenerating muscle fibres were present in it, it had evidently formed in place of destroyed muscle tissue.

In the legs of the remaining animals killed during the first 16 days after the fracture, muscle fibres undergoing degeneration were rare, but regenerating fibres were very numerous, growing through areas of young connective tissue.

We have little doubt that the amount of muscle tissue which was destroyed after suffering injury at the operation was much less in the dietetically normal animals than in the partially deficient, but no quantitative estimate was possible. It is certainly true that the amount of hyperplastic connective tissue formed in the limbs of the partially deficient animals was very much greater than in those on the normal diet, this difference must evidently reflect either a greater quantity of muscle destroyed in the partially deficient animals, or a greater quantity regenerated in the normals, or most probably both.

In dietetically normal animals killed 3 months or more after the operation, there was little or no connective tissue beyond the normal, and no regeneration in progress, the injured fibres had, apparently, long since been restored to the normal condition. This was, of course, in sharp contrast with the partially deficient animals in which the hyperplastic connective tissue was, at corresponding times, at its fullest development, in which there had been very much less regeneration of muscle fibres, and in which such regenerates as did occur failed to attain anything like the normal size.

Aetiology

It is natural to attribute the oedema, the exudates, and the muscular degenerations, to the injury inflicted by the fracture forceps. This is supported by the following figures.

In Exp. 9, in which we studied the development of the connective tissue hyperplasia, killing sixteen animals at various times after the operation, if the formation of the hyperplasia were a consequence of the operation, it would not be expected to occur until several days after it. This was the case, for it had not appeared in animals killed at 3 days, was beginning in those killed at 5 and 8 days, but was large at and after 11 days.

Again, if the hyperplasia were a result of the operation, it should have occurred in the operated and not in the unoperated limbs. Of the sixteen animals in Exp. 9, thirteen showed hyperplasia in the operated leg, and the remaining three, which were those killed at 3 days after the operation, showed in the muscles of the operated legs the changes which precede the formation of hyperplastic connective tissue. Of the sixteen unoperated legs, thirteen showed no sign of hyperplasia, while some hyperplasia was present in three (19%). In two of these it was much less than on the operated side. In Exps. 5 and 6, nineteen animals on partially deficient diets suffered the operation on one side. There was formation of hyperplastic connective tissue in at least seven of the nineteen operated legs (37%). In the same experiments, eight other animals were similarly dieted but not operated (groups 5 and 6). Among the thirty-five legs, made up of two from each of these eight and of the nineteen unoperated legs of the animals mentioned above, connective tissue hyperplasia developed in at least eight (23%). In Exps. 3 and 4, which included nineteen animals kept on diets partially deficient in vitamin C, some connective tissue hyperplasia occurred in at least eight out of twenty-six operated legs (31%), and in at least one out of twelve legs which were not operated (8%). In this experiment the operation was performed 2 weeks before the diet was made partially deficient in vitamin C, and during this period repair was proceeding on a fully supplemented diet, nevertheless, the partial deficiency, late though it began, had the effect just stated.

We conclude that (1) the connective tissue hyperplasia developed more readily in operated than in unoperated legs, but (2) it could develop in legs which had suffered no operative interference.

The hyperplasia never developed in the legs of animals receiving 10 mg. of ascorbic acid daily.

The evidence clearly points to an inability of the musculature of the partially vitamin C-deficient guinea-pigs to regenerate after injury, and probably indicates

a greater susceptibility to injury in these animals, than in those supplied with adequate quantities of the vitamin. Why, then, was there in some cases a connective tissue hyperplasia in unoperated limbs? The only answer we can make to this is to suggest that the muscles, which the vitamin deficiency has made more liable to injury, can be so affected by traumatic agents, which would have much less effect on normal limbs, as to bring about the changes which we described above. Possible occasions of such injury were the animals' kicking during the taking of radiographs, in handling during administration of ascorbic acid, etc.

We have examined the blood vessels in sections through the legs of partially vitamin C-deficient and dietetically normal animals. In the operated legs of partially deficient animals the arteries especially were affected. The changes could be recognized in the animals killed at 3 days, and were seen in full development in those killed at 5 days. At this time, and later, many of the vessels were empty of corpuscles, or nearly so, especially the arteries. Again, chiefly in the arteries, the endothelial cells were swollen by vacuoles developed in the basal parts of the cells, between the nuclei and the internal elastic membrane. The vacuoles cause the cells to project into the lumen of the vessel (Pl. 5, figs 24, 25). Small arteries may be blocked by projecting, and even desquamated, endothelial cells. Delicate strands seen in the sections crossing the lumen from side to side were difficult to interpret with certainty, they seemed to be the walls of very greatly expanded endothelial vacuoles. Vacuoles might also appear beneath the internal elastic membrane and among the smooth muscle fibres. Occasionally, the vacuolation here became so intense that the muscle fibres were loosened up and separated from one another (Pl. 5, fig. 26), rarely, the disintegration process might go so far as a complete breakdown of the cross-section of the vessel (Pl. 5, fig. 27), which might then be histologically recognizable only by tracing a connexion, through serial sections, with its more normal parts. The veins usually appeared normal, and contained blood, with vacuolate and swollen endothelium. Lymphatics were numerous and often greatly expanded, mitotic figures were often seen in their walls.

Examination of the blood vessels in those legs of guinea-pigs which were killed or died at times ranging up to 3 or 4 months after the operation, and which showed hyperplasia of the connective tissue, also showed that the conditions just described in animals killed during the first 18 days after the operation had persisted. The hyperplastic connective tissue was always nearly avascular, and most of the blood vessels (especially the arteries) in or near it were empty or nearly so, with vacuolate and swollen endothelium. When, on the other hand, there was no development of hyperplastic connective tissue, the blood vessels were normal.

Comparison with the operated legs of dietetically normal animals, and especially with those killed in the first 3 weeks, showed that it would be almost true to say that the vascular changes just described did not occur in these animals. In some specimens, however, especially in those whose legs showed any large accumulation of connective tissue, empty vessels, especially arteries, with vacuolate endothelium, could be found. But this was far less frequent than in the partially deficient animals and, when present, was less severe.

The present experiments do not enable us to decide whether the partial deficiency of vitamin C, in preventing adequate regeneration of injured muscles and causing

their replacement by connective tissues, acted directly, by increasing the sensitivity of muscle fibres to injury and/or by reducing their power of recovery and regeneration, or indirectly, as by causing interference with the vascular supply. Le Gros Clark & Blomfield (1945) found that interruption of the arterial supply to the muscles of otherwise normal animals led to degeneration of the muscle fibres and their temporary replacement by connective tissue, when an adequate collateral blood supply had been established, regeneration re-established the muscle and the excess connective tissue melted away. This suggests that the primary effect, in our partially deficient animals, may have been on the blood vessels. The use of the fracture forceps in the operation inevitably injured the muscles by squeezing them, causing haemorrhages by rupturing capillaries and other vessels. Haemorrhagic areas are very common in the muscles of our partially deficient animals, but the degenerative changes we have described often occurred in their absence. The vascular stagnation resulting from the rupture of many small vessels, and the oedematous condition of the muscles, might be expected to cause such anaemia as was presumably responsible for the muscular degeneration in Le Gros Clark & Blomfield's experiments, and might also take the blame for the closely similar changes in our material. However this may be, the blood vessels or at least the arteries, are apparently more liable to injury in the partially deficient animals than in the dietetically normal controls. Meyer, in scorbutic animals, mentions changes in blood vessels similar to those we have described, but unrelated to any known injury and to hyperplastic connective tissue.

Our guinea-pigs were not supplied with any special supplement of vitamin E, and a number of authors (Einarson & Ringsted, 1938, Evans & Burr, 1928, Evans, Emerson & Telford, 1938, Olcott, 1938, Pappenheimer, 1939, 1943) have described degenerative muscular changes, in various species, when deprived of this vitamin. In some respects the changes described above resemble these descriptions, and the question arises whether the changes in our vitamin C-partially deficient animals could have been due to lack rather of vitamin E than of vitamin C. A number of facts tell against this idea. First, the changes in our material were local, whereas those caused by vitamin E deficiency are widely spread through practically the whole skeletal musculature. Secondly, authors describing the muscles in vitamin E deficiency do not mention the wholesale local replacement of muscles by hyperplastic connective tissue. Thirdly, and most convincingly, ascorbic acid protected our animals against the changes we have described, and would not do so against those of vitamin E deficiency.

SUMMARY

1 In partial vitamin C deficiency the formation of callus at fractures of the fibula was slower, and the amount of callus formed was at first less than in dietetically normal animals.

2 Whereas in normal animals the callus later consolidated into compact bone by thickening of the trabeculae, in the partially deficient animals this did not occur, the callus might become extremely extensive, covering the whole diaphysis (see the second paper of this series), but always retained a lightly built, trabecular structure.

3 In a large proportion of animals kept on partially vitamin C-deficient diets, the knees became stiff and could be bent only painfully and with difficulty. Evidence is presented indicating that this condition was caused at least in part by local histological changes.

4 The manner of fracturing the fibula caused some injury to muscle, and damaged muscle fibres degenerated. In dietetically normal animals they quickly regenerated. In partially vitamin C-deficient animals, much more muscle degenerated than in dietetically normal animals, and the muscular tissue so lost was replaced by large masses of hyperplastic connective tissue and did not regenerate. A large part of the limb musculature might thus disappear. The process of degeneration of the muscle fibres, and the development of the hyperplastic connective tissue, are described.

5 The degeneration of muscles and their replacement by hyperplastic connective tissue occurred more readily in operated legs (in which the muscles were certainly damaged) than in unoperated, but it was also seen in unoperated limbs, though less frequently. It is suggested that the muscles of partially vitamin C-deficient animals are more liable to injury than those of normal animals, and can be damaged by traumatic agents which do not affect those of normals. Possible occasions of such injury were the animal's kicking when being anaesthetized before radiographs were taken.

6 The hyperplastic connective tissue was always avascular or nearly so. Examination of the blood vessels, during the period of muscle degeneration, showed abnormalities especially in the arteries, and these are described. The vessels were found to be in the same condition in the hyperplastic connective tissue of animals which lived for months after the operation. The evidence does not permit us to say whether the replacement of muscles by connective tissue reflected a direct effect of the partial deficiency of vitamin C on the muscle fibres and their ability to regenerate, or an indirect effect through the blood vessels, a certain resemblance of the histological picture to that described by Le Gros Clark & Blomfield, after experimental interruption of the arterial supply, suggests the latter.

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EXPLANATION OF PLATES

List of abbreviations used in plates *cal* callus, *cart* cartilage, *fib* fibular, *fract* position of fracture *nb* new bone, *peri* periosteum, *tib* tibia ✓

PLATE 1

- Fig 1 Normal animal Stages in the repair of the fractured fibula, from Exp 6, group 1
- Fig 2 Partially vitamin C deficient animal Stages in the repair of the fractured fibula, from Exp 6, group 4
- Fig 3 Normal animal Stages in the repair of the fractured fibula from Exp 5, group 1
- Fig 4 Partially vitamin C deficient animal Stages in the repair of the fractured fibula, from Exp 5, group 3

PLATE 2

- Fig 5 Normal animal Fibula, 9th day after fracture, showing cartilage (lower right) in the developing callus Giemsa $\times 35$ From Exp 1
- Fig 6 Normal animal Fibula 9th day after fracture No callus at fracture but new bone on fibula at some distance from it, and on tibia Tibia and its marrow on left Haematoxylin and eosin $\times 17$ From Exp 1
- Fig 7 Normal animal Fibula 14th day after fracture, showing callus Azan $\times 38$ From Exp 1
- Fig 8 Normal animal Fibula 16th day after fracture Haematoxylin and eosin $\times 25$ From Exp 1

PLATE 3

- Fig 9 Normal animal Fibula 101st day after fracture Bracket indicates consolidated callus Haematoxylin and eosin $\times 24$ From Exp 6, group 1
- Fig 10 Partially vitamin C deficient animal Fibula 14th day after operation 40th day of experiment Extensive resorption and no callus at site of fracture new subperiosteal bone forming on both stumps Azan $\times 27$ From Exp 9
- Fig 11 Partially vitamin C deficient animal Fibula, 18th day after fracture and 45th day of experiment Azan $\times 58$ From Exp 5, group 4

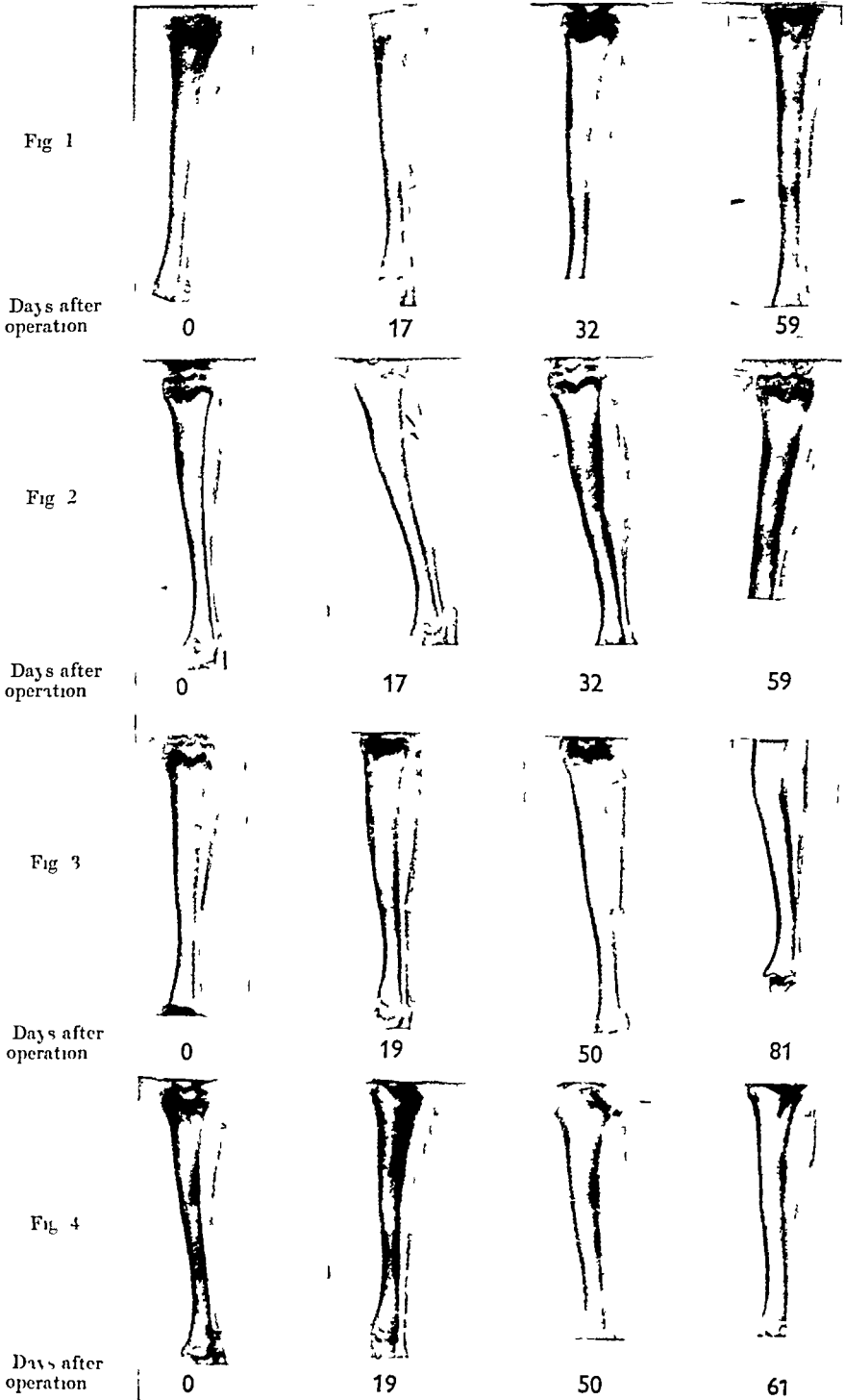
- Fig 12 Partially vitamin C deficient animal Fibula and part of tibia, 61st day after fracture and 85th day of experiment Haematoxylin and eosin $\times 12$ From Exp 6, group 4
- Fig 13 Partially vitamin C deficient animal Fibula and part of tibia, 93rd day after operation, 120th day of experiment Haematoxylin and eosin $\times 15$ From Exp 5, group 4

PLATE 4

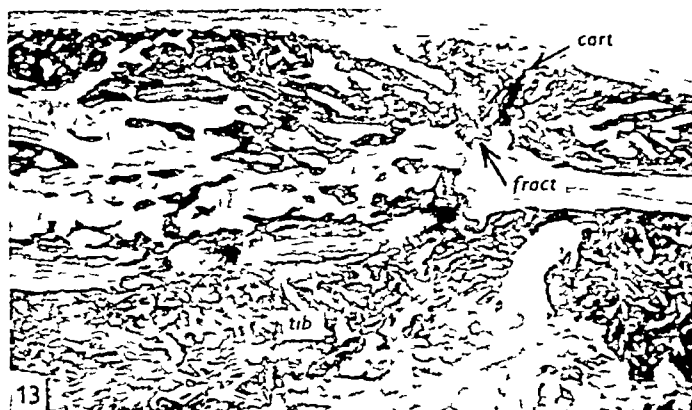
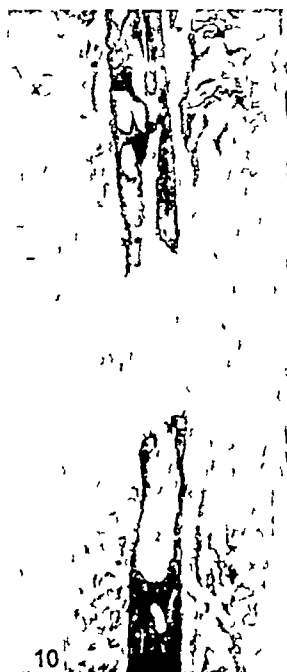
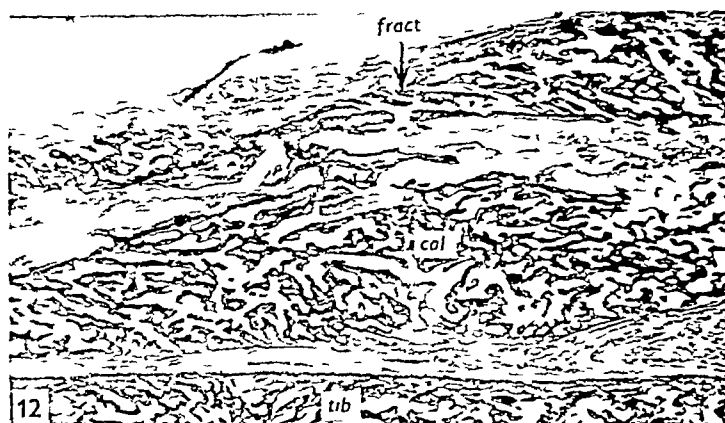
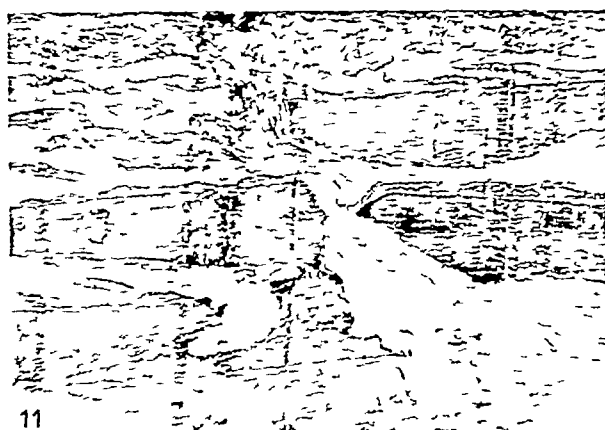
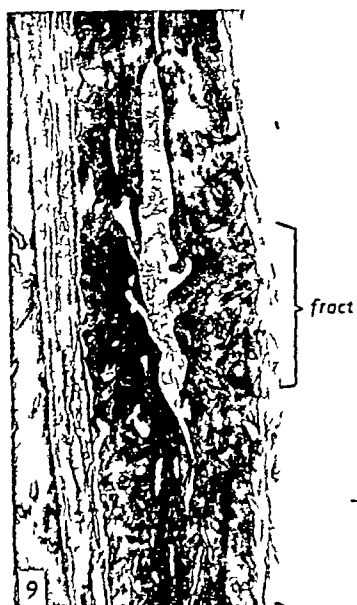
- Fig 14 Partially vitamin C deficient animal Fibula and part of tibia (right), 81st day after operation, 66th day of experimental diets Haematoxylin and eosin $\times 20$ From Exp 4, group 4
- Fig 15 Partially vitamin C deficient animal Part of transverse section of shank, showing hyperplastic connective tissue, 102nd day after operation and 87th day of experimental diets Haematoxylin and eosin $\times 17$ From Exp 3, group 3
- Fig 16 From the same specimen as fig 15, showing hyperplastic connective tissue Azan $\times 60$
- Fig 17 Partially vitamin C deficient animal, 81st day after operation, 67th day after beginning experimental diets An area of tibial surface where the periosteum has dissolved in the surrounding hyperplastic connective tissue Haematoxylin and eosin $\times 175$ From Exp 4, group 4

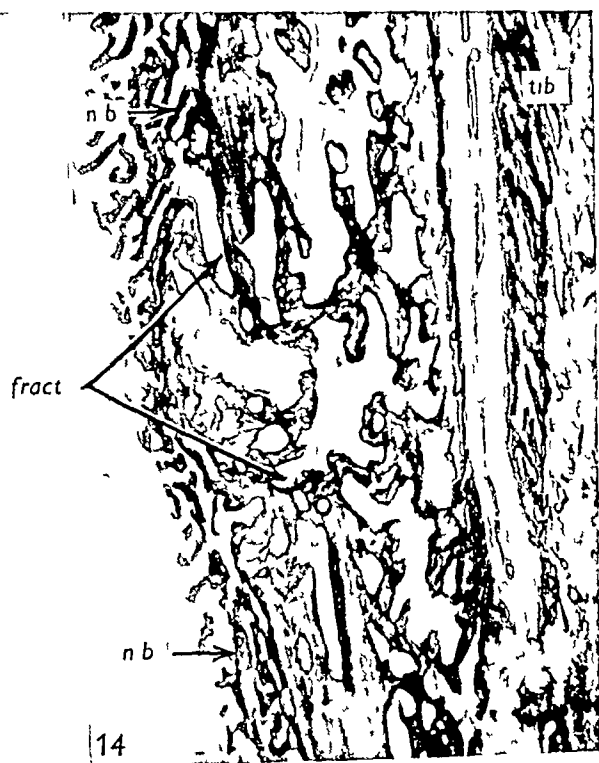
PLATE 5

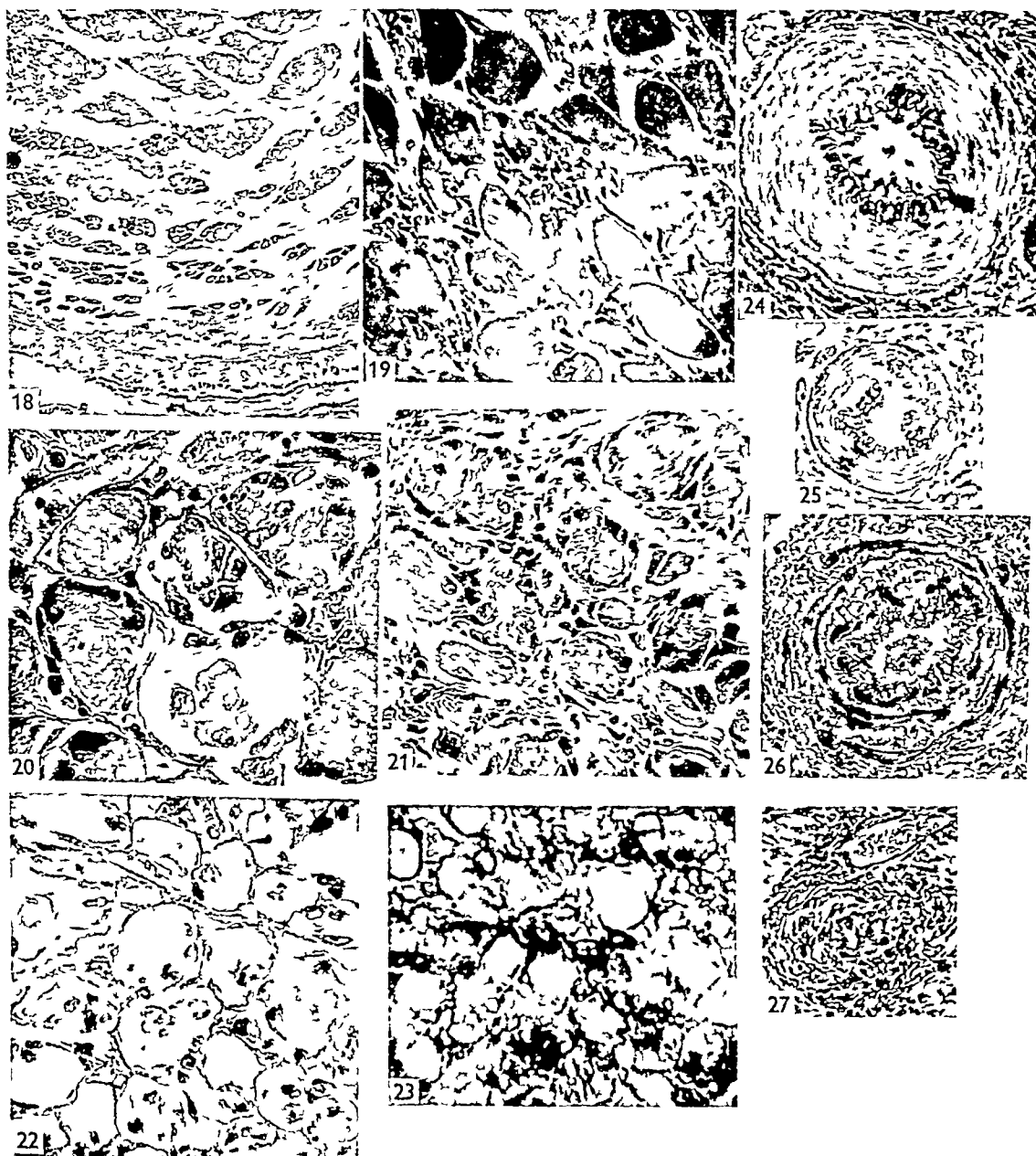
- Fig 18 Partially vitamin C deficient animal An oedematous muscle, and, below, the fibular periosteum, 4th day after operation and 29th day of experiment Haematoxylin and eosin $\times 135$ From Exp 9
- Fig 19 Partially vitamin C deficient animal Early degeneration of muscle fibres which show neither nuclei nor myofibrils, 4th day after operation, 29th day of experiment Haematoxylin and eosin $\times 580$ From Exp 9
- Fig 20 Partially vitamin C deficient animal Degenerating muscle fibres showing 'pitting' by small vacuoles, and fragmentation of fibres, 6th day after operation, 31st day of experiment Azan $\times 600$ From Exp 9
- Fig 21 Partially vitamin C deficient animal Degenerating muscle fibres under attack by mono and multi nucleate cells, 6th day after operation and 31st day of experiment Azan $\times 440$ From Exp 9
- Fig 22 Partially vitamin C deficient animal Empty envelopes of muscle fibres occupied by wandering cells, 6th day after operation, 31st day of experiment Haematoxylin and eosin $\times 575$ From Exp 9
- Fig 23 Partially vitamin C deficient animal The hyperplastic connective tissue, from the same specimen as fig 15 Azan $\times 745$ From Exp 3, group 3
- Fig 24 Partially vitamin C deficient animal An artery with vacuolation of the endothelium, 12th day after operation, 37th day of experiment Haematoxylin and eosin $\times 210$ From Exp 9
- Fig 25 Partially vitamin C deficient animal An artery with vacuolate and thickened endothelium, 4th day after operation, 29th day of experiment Haematoxylin and eosin $\times 210$ From Exp 9
- Fig 26 Partially vitamin C deficient animal An artery showing vacuolation in both endothelium and connective tissue of the wall, 19th day after operation, 44th day of experiment Haematoxylin and eosin $\times 210$ From Exp 9
- Fig 27 Partially vitamin C deficient animal Remains of a degenerate artery, only recognizable by tracing through serial sections, 15th day after operation, 40th day of experiment Haematoxylin and eosin $\times 210$ From Exp 9











REVIEWS

Human Embryology and Morphology By SIR ARTHUR KEITH 6th ed (Pp xii+690, 578 figures, demy 8vo, 40s net) London Edward Arnold and Co

Sir Arthur Keith in the Preface to this 6th edition of his well-known book on Morphology and Embryology compares it with the 1st edition of 1901. He notes that in the intervening years the centre of embryological and morphological research has shifted from Germany to the United States, and he generously acknowledges the debt that the book owes to the Carnegie Laboratory in Baltimore. It is a pleasure to notice too his recognition of the immense amount of original work that constitutes Prof. Frazer's *Manual of Embryology*, to which also the present book owes much.

Sir Arthur gives an interesting review of his own changed attitude with regard to the teaching of Embryology. At one time 'clinical utility was the criterion employed', but now 'everything which seemed to throw new light on our understanding of the human body was welcomed to my pages'. But he has remained firm in his guiding principle 'that Embryology becomes a profitable study only when we interpret its events in the light of evolution'.

This principle has clearly been adhered to, for almost no matter where one opens the book a reference is found to lower vertebrates.

The book is hardly recognizable as the child born in 1901. Like the rest of us it has suffered from the war, and the binding, paper and print though surprisingly good are not equal to those of the 1st edition. The greatest change is in the 'Notes and References' appended to each chapter, and these are even increased from the 5th edition of 1933, comprising now nearly 50 pages of close-set type. Still, there are familiar features, the same friendly conversational style of writing, the same technique of line and stipple in the figures, many of the original blocks still being used.

Many of the figures have been borrowed, and not all lend themselves to the style of technique referred to. Several of the figures are printed so darkly that it is hard to make out the points they are meant to illustrate.

The book brings to mind the question as to how far students are prepared to read outside their set books. If they are so inclined, here is the book to show them where to browse, but, the curriculum being what it is and the tendency being to cut down to a bare minimum the time allotted to Anatomy, it is doubtful if many of them will have time to reap the rich harvest that Sir Arthur has prepared for them. There will be many sources of information that the students' teachers may have overlooked, but I suggest there are few important papers that have escaped Sir Arthur's comb. He is to be congratulated on the care with which he has ensured that the book shall be up to date. The task of collecting such a multitude of references is one which many a younger man would have shirked, but Sir Arthur has not only achieved this but has found the time and energy to give us his own opinions on many points, and has not hesitated to show us how these have changed with the passage of time and in face of new facts.

The child of 1901 has now become a book of reference that may be profitably studied both by students and their teachers.

C. M. WIST

Living Anatomy. A Photographic Atlas of Muscles in Action and Surface Contours
By R. D. LOCKHART, M.D., CH.M. (Pp 71, 149 figures, royal 8vo, 12s 6d net)
London. Faber and Faber

The aim of this book 'to awaken the student's interest in studying, literally at first hand, muscles in action in the living body' is excellent. The static representation by still photography of a dynamic subject is not easy, but by selecting examples where the

muscles are in isometric contraction, the author has presented the subject well. A brief description is given with most of the 149 photographs and the student should have little difficulty in recognizing similar features in his fellows and himself.

A few of the positions adopted by the subjects are unusual and it would have been better to omit them. The inclusion of Harvey's experiments to demonstrate the action of valves in veins is an excellent contribution, but the photographs should be placed in the book so as to give the impression of the action of gravity.

The photography is, on the whole, so good that isolated pictures which are not perfect stand out in contrast. This is particularly noticeable in Fig. 35 where, in spite of the author's warning in the Introduction, the lower border of the trapezius is shown running in the direction of the acromion process.

The book is worthy of its aim and should be very useful to medical students and others who must be able to recognize muscle activity in the living.

J. WHILLIS

IN MEMORIAM

CHARLES JOSEPH PATTEN, M A , M D , Sc D , F R A I
Emeritus Professor of Anatomy, University of Sheffield

Charles Joseph Patten died on 13 June 1948, after a very brief illness, at his home in Farnham, Surrey, where he had moved from Sheffield only a year previously. In his later years he suffered from a severe affection of his eyes. By his death the Anatomical Society has lost one of its oldest members. He was the son of Richard Patten, of the Court of Exchequer, Ireland, and was born at Ballybrack, Co. Dublin, in 1870. Patten was educated at the High School, Dublin and later at Trinity College, Dublin, where he took a triple first in the Senior Moderatorship with a gold medal in Natural Science. He graduated in 1896 and became a Demonstrator in Anatomy in Cunningham's department at Trinity College. His research during his tenure of this office included studies of the topographical relations and surface anatomy of the heart, lungs and pleural cavities.

In 1901 he was elected Professor of Anatomy in the University of Sheffield (then a university college) in succession to Christopher Addison, now Lord Addison of Stallingborough. On retirement from his chair in 1935 he was given the title of Professor Emeritus. At Sheffield he developed the beautiful museum in the Anatomy department, his anatomical studies, on which he contributed many papers to the meetings of the Anatomical Society, were mainly in the field of physical anthropology, particularly variations in the osteology of the skull in primates and anomalies in human myology. He took a keen interest in finding the best methods for the preservation of the cadaver, his 'dry-tank' preservation has stood the exacting test of time. But his greatest interest over many years was the study of bird life and it was as an ornithologist that he became best known to the general public through his writings, lectures and broadcasts. Before leaving Sheffield he gave his excellent collection of bird skins to the Weston Park Museum, Sheffield. He had a great affection for birds and studied them mainly as a naturalist, it was their ways and habits, their nesting and particularly the problem of their migration that appealed most to him. He spent many weeks during university vacations in the off-shore lighthouses around the Irish coast studying bird migration and he wrote numerous papers and two books on bird life, one of which *The Aquatic Birds of Great Britain*, published in 1912, is a standard work.

Patten acted as external examiner in anatomy at various times in the Universities of Wales, Birmingham, Leeds and Manchester and the Queen's University of Belfast. Most notable of Patten's qualities were his imperturbable cheerfulness, his generosity, tolerance, ready help and pronounced sense of humour. He had the gift of excellent mimicry and his lectures were enriched by his imitation of the sound and song of numerous birds. He was a devoted disciple of Charles Darwin whom he literally worshipped. He will be missed by a wide circle of friends. He married Mabel Elizabeth, daughter of Canon W. J. King, and is survived by his widow and a son and daughter, to whom we extend our deepest sympathy.

F. D.

M. A. MacC.



CHARLES JOSEPH PATTEN

ARTHUR ROBINSON, M D , F R C S Edin and Eng, LL D
1862-1948

Emeritus Prof Arthur Robinson, who occupied the Chair of Anatomy in the University of Edinburgh from 1909 to 1931, died at Eastbourne on 3 December 1948, aged 86

Arthur Robinson was born at Manchester in 1862, and he graduated M B , C M , with honours, at Edinburgh in 1883 After a brief apprenticeship in the teaching of Anatomy at Surgeons' Hall, he was appointed Demonstrator in the University Department by Prof Turner Next year he accepted a call to Owens College, Manchester, as Assistant to Prof Morison Watson, and in 1888 he became Senior Demonstrator and, later, Lecturer in the Victoria University under Prof A H Young

The 70's and 80's were indeed vintage years for Edinburgh anatomists Robinson himself (graduating two years before Harold J Stiles—a surgeon-anatomist of the first rank) came in the midst and was the last survivor of an unparalleled group that included Morison Watson, J H Scott, D J Cunningham, A. H Young, Johnson Symington, Arthur Thomson, David Hepburn, A M Paterson, J T Wilson, Robert Howden, James Musgrove, T H Bryce and Edward Fawcett—all of them Turner's Assistants and all of them professors in due season

In 1896 the Medical School of The Middlesex Hospital decided to appoint a whole-time Lecturer to succeed Bland Sutton, and Robinson, with the reputation he had made in Manchester as teacher and investigator, was selected for that new post His success in reorganizing and extending the Department at The Middlesex Hospital led to his appointment four years later to the Chair of Anatomy in King's College, and at similar short intervals he moved on—first, in 1905, to the University of Birmingham (where he was also Sub-Dean of the Faculty of Medicine), and finally to Edinburgh in 1909 to occupy the Chair left vacant by the early death of D J Cunningham In all these posts, while continuing to investigate problems in his chosen fields of research, he abundantly displayed his administrative talent and his powers as a teacher

In London, Robinson not only added to his reputation as an effective teacher—his classes in preparation for the 'Primary Fellowship Examination' spread his fame far and wide—but he also took an active part in the development of anatomical teaching in relation to the University of London He served as Secretary of the Board of Intermediate Medical Studies, and he was particularly concerned with the movement that resulted in the formation of the Board of Studies in Human Anatomy and Morphology by the University Senate

Robinson's association with Turner, who had himself made numerous contributions to the subject of placentation, and with A H Young, with whom he collaborated in several morphological studies, doubtless influenced his choice of Comparative Embryology and Vascular Morphology for his principal researches During his Manchester period he was actively engaged in both of these fields as the list of his published papers testifies, in addition to other developmental and morphological studies His first paper appeared in 1887, and in 1890 he was awarded the

Gold Medal of the University of Edinburgh for his M D thesis 'Observations on the development of two rodents' His interest in the development and morphology of veins, on which he came to be recognized as an authority, dates from 1891, when he contributed a paper to the first volume of *Studies in Anatomy* from Owens College But the outstanding papers of the Manchester period are the two that appeared in the *Quart J micr Sci* on early developmental stages These papers gave him his initial reputation as an embryologist, and it may be noted that he was asked in 1894 to give a Course of Lectures on Comparative Embryology as deputy for Milnes Marshall

That reputation was firmly established by his better-known work on the Comparative Anatomy of the Placenta (*J Anat, Lond*, 1904, 38), based on three lectures which he delivered in 1903 as Hunterian Professor of the Royal College of Surgeons of England

In 1898 Robinson contributed several chapters to the second edition of the text-book edited by Henry Morris of the Middlesex Hospital, and in 1902 he resumed his collaboration with A H Young by contributing with him to the first edition of the *Text-Book of Anatomy* edited by D J Cunningham, in which they appeared as joint-authors of the Sections on 'General Embryology' and the 'Vascular System' When Robinson succeeded Cunningham in the Edinburgh Chair he also succeeded him as editor of the *Text-Book* and became responsible, too, for successive editions of Cunningham's *Manual of Practical Anatomy* He at once introduced the B N A system of nomenclature on the grounds 'firstly, and chiefly, because it is more regular and definite than that hitherto in use, and, therefore, it tends to inculcate greater definiteness of idea and statement Secondly, because it has been very generally adopted in Canada, Australia and America' He thus brought to an issue the apparently interminable terminological arguments which were ultimately settled, so far as British anatomists are concerned, by the adoption of the Birmingham Revision in 1933

These editorial labours, together with the heavy responsibilities of a large Department, took up a great deal of his time Nevertheless, with his abounding energy, he continued to pursue the developmental problems that interested him, and his paper on 'The formation, rupture and closure of ovarian follicles' (1918) was a major contribution to the subject, in which he described the formation of 'secondary liquor folliculi' as the immediate cause of the rupture of the follicle

In 1912 Robinson had been elected a Fellow of the Royal College of Surgeons of Edinburgh, and in 1920 he was Struthers Lecturer of that College He took as his subject 'Prenatal Death', and he reviewed the evidence for the varying viability of the zygote in different animals with reference to the high rate of early abortion On his transfer to Edinburgh he had been appointed Chairman of the William Ramsay Henderson Trust, and he was largely responsible for the series of Lectures inaugurated in 1924 by Prof Elliot Smith who delivered a discourse on 'The Old and the New Phrenology'

Robinson was a Fellow of King's College, London, and in 1924 he was elected to the Fellowship of the Royal College of Surgeons of England as a Member of twenty years' standing The Royal Society of Edinburgh awarded him the Neill Prize for the period 1925-7 for his 'Contributions to Comparative Anatomy and

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AGE CHANGES REVEALED BY CARBONYL REAGENTS IN TISSUE SECTIONS

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The histochemical reactions obtained with the two carbonyl reagents, fuchsin sulphurous acid (FSA) and 2,4-dinitrophenylhydrazine (DNPH), have been previously investigated in the organs of the rat (Albert & Leblond, 1946). A study of these reactions in mice tissues showed a widespread distribution similar to that found in the rat. Furthermore, the observations carried out in mice revealed that the intensity of the staining varied with the age of the animals. Thus, the carbonyl reactions present in sections of sex glands and accessory sex organs, such as seminal vesicles, uterus and mammary gland, usually became prominent at puberty, but tended to disappear in old animals.

This effect of ageing suggested that the staining intensity of many tissues varied with their physiological activity, and, therefore, the water-insoluble carbonyls (plasmalogens?) detected with FSA and DNPH may have more biological importance than hitherto believed.

MATERIAL AND METHODS

Age differences in the staining with FSA and DNPH were examined in mice of the high-cancer 'A' and low-cancer 'C57' strains at the ages of 1 day, 1, 2-3, 3-9, 11, 15 and 16 months. Four mice (two males and two females) of each strain were used per group. In the 15-month-old group, only 'A' mice were available. In addition, similar studies were carried out in four 8-10-month-old female mice of the high-cancer 'C3H' strain which bore mammary tumours.

A fairly complete study of the distribution of the reaction in almost all organs and tissues was carried out in several of the animals, but only the following tissues were systematically examined in all adrenals, liver, kidney, heart, sex glands and accessory sex organs.

The technique employed in the staining of tissues with FSA was essentially that of the 'plasmal reaction' (Lison, 1936). This technique consisted of fixing the tissues in neutral formalin, washing in water, cutting frozen sections, and staining them with FSA after passage through a saturated solution of mercuric chloride.

Since erratic results and patchy staining were obtained in some of our earlier tests, experiments were carried out to determine the effects of various factors on the stainability of tissues with FSA. It was found that (1) varying the duration of formalin fixation from 2 to 4, 24 or 48 hr did not alter the intensity and patchiness of the stain, (2) no staining occurred if sections were cut after fixation and immediately taken through mercuric chloride and FSA without washing, (3) if after fixation the sections were allowed to stand in water in a shallow open dish overnight, intense and uniform staining resulted.

These observations suggested that oxidation by air may have played a role in the reaction. Indeed, if the washing of the sections was carried out in oxygen-free distilled water using completely filled, tightly stoppered bottles, very little or no reaction was observed with FSA. Conversely, the addition of 1-2 drops of 3% hydrogen peroxide (Merck, U S P) per c c of tap water produced an intense and regular stain. Under these conditions, washing for $\frac{1}{2}$ hr instead of 12-24 hr was sufficient to ensure a good stain. Gérard (1935) had previously noted that the 'plasmal reaction' was not obtained after formalin fixation, unless subsequent oxidation was carried out.

It was concluded that staining after formalin fixation required some degree of oxidation using either the oxygen of the air or H_2O_2 , each producing a regular and apparently identical staining pattern. It was also noted that sections, after such an oxidation, stained even if washing in mercuric chloride had been omitted. This treatment, however, frequently intensified the stain, but did not alter its distribution.

The oxidation provided by exposure to the air was adopted for routine work, since it was milder and, therefore, less likely to oxidize alcoholic to carbonyl groups in the sections. After a 48 hr fixation in 10% neutral formalin and 24 hr washing in running water, the tissue blocks were sectioned at 10μ on the freezing microtome. The sections were then washed in distilled water in shallow dishes for about 24 hr prior to staining. During the night, they were kept in the cold room. The washed sections were placed in a saturated solution of mercuric chloride for 3 min, rinsed, left for 5 min in FSA, washed in two solutions of sulphurous acid and mounted in glycerine-gelatine. A positive reaction was indicated by a purple colour. This colour was found to be very stable, since it has persisted for over a year in our material. Special care was taken to carry out the various operations simultaneously for all experimental groups, thus ensuring a uniform technical procedure.

The method used for staining sections with DNPH has been described previously (Albert & Leblond, 1946). The same precautions for fixation and washing as described above were taken in this procedure.

While most of the work was performed on formalin-fixed tissues, the FSA reaction was also examined in the fresh, unfixed tissues of two male hybrid albino mice and three female mice of the 'C3H' strain. These tissues were quickly removed from the animals, sectioned at $20-30\mu$ on the freezing microtome, placed immediately in a saturated solution of mercuric chloride for 3 min and stained with FSA for 5 min. Control slides were placed in FSA without pre-treatment with mercuric chloride.

In all cases the staining intensity of histological structures was rated in four different categories, namely, very intense, intense, moderate, and slight, and will be described below.

RESULTS OBTAINED WITH FIXED TISSUES

The general distribution of the FSA and DNPH reactions in the formalin-fixed tissues of 'A' and 'C57' mice was similar to that previously reported for the rat (Albert & Leblond, 1946). However, the staining intensity of most organs, especially kidney, adrenal cortex, corpus luteum and interstitial cells of the testis and ovary, was greater in mice than in rats. With both species there was a strict parallelism in the distribution of the FSA and DNPH reactions (Pl 1, figs 1, 2).

Endocrine glands

The staining of the *adrenal gland* varied markedly with age, although in all animals there was a definite staining of the cortex, but little or no staining of the medulla. In 1-day-old mice, the cortex stained intensely and rather uniformly throughout. There was no division of the cortex into the three zones normally found in older animals, the only suggestion of zoning being a less intense stain in a few superficial cells. At the age of 1 month, the adrenal cortex had become divided into three zones, of which the fascicular alone stained as intensely as did the whole cortex of the newborn. Both the glomerular and reticular zones were thin and stained slightly.

At the age of 3 months, the staining intensity of the fascicular zone reached its maximum. The thin glomerular and the thickened reticular zone stained only slightly, although more intensely than the medulla (Pl 1, figs 1, 2, Pl 2, figs 6, 7). In the 'A' females of this age group there was a band of cells distended by a large fatty vacuole (signet-ring cells) located between the reticular zone and the medulla. The fatty content of the large vacuole of these cells stained moderately, though it often fell out during the preparation of the sections (Pl 2, fig 6). The small ring of cytoplasm contained minute granules staining intensely. These signet-ring cells were not found in the adrenals of the 'C57' animals of corresponding age (Pl 2, fig 7). The reticular zone in this strain was uniformly thinner than in the 'A' animals, being hardly distinguishable in some cases. Furthermore, in the 'C57' animals, the cell cords of the fascicular zone were less regularly orientated, and the limit between the zones was not sharp, as typical fascicular cells extended into the reticular zone.

In the older age groups of both strains, the irregularity of the cell cords in the cortex became quite pronounced. The glomerular zone so lost its distinctness that it was unidentifiable in some of the oldest animals. In the fascicular zone, the cell cords stained less intensely and frequently lost their radial arrangement. Furthermore, a fairly intense reaction could usually be seen in the inner half of the fascicular zone, while its outer half reacted poorly (Pl 3, fig 17). In the oldest animals there was a breaking down of the inner fascicular cords into irregular, deeply staining masses and granules (Pl 3, fig 16). The reticular zone was also less regularly arranged than at 3 months, and its vascularization was increased, especially at its junction with the medulla. With age, the fatty globules described at the outer limit of the medulla in the 'A' strain disappeared. Finally, pigment was found in the reticular zone of mice of the later age groups in both sexes of both strains, especially in the 'C57'. These pigment masses appeared to be identical with those found in brown degeneration (Cramer & Horning, 1937, Lacassagne & Raynaud, 1937, Leblond & Nelson, 1937, Cook & Kennaway, 1940, Blaisdell, Gardner & Strong, 1941, Burrows, 1945, Tobin & Birnbaum, 1947).

In the *testis* of all but the oldest animals, the slight reaction found in the tubules contrasted with the good staining of the interstitial cells. These cells showed a moderate reaction as early as the first day of life (Pl 4, fig 21). The reaction was fairly intense at 1 and at 3 months (Pl 4, fig 22). In the 15-month-old mouse, however, there were only a few or even no cells giving a definite purple reaction in the interstitial spaces. The staining intensity of the Leydig cells, when any of them could

be identified, was much less than in the younger animals (Pl 4, fig 23). On the other hand, numerous irregular pigment masses were scattered throughout the interstitial spaces where the natural brown colour of the pigment contrasted with the light purple background in the FSA-stained slides.

In the *ovary* of all but the oldest animals, the slight reaction found in the contents of the Graafian follicles contrasted with the good staining of the thecal, interstitial and luteal cells. Thus in the follicles, the granulosa cells gave little or no reaction, the oocytes showed fine, discrete granules with a fairly intense colour, while the follicular fluid did not react at all. The thecal cells gave an intense colour, but in places their reaction was moderate (Pl 2, figs 8, 9). The interstitial cells showed a moderate reaction at 1 month, a very intense one at 3 months (Pl 2, figs 8, 9), and a decreased reaction at the later age intervals, especially at 15 months, when they either stained slightly or not at all (Pl 3, figs 13, 15). The atretic follicles sometimes showed a few intensely staining patches corresponding to groups of lipid-laden cells. There was a wide range in the staining intensity of corpora lutea. In the 3-month-old animal of the 'C57' strain, for instance, intensely staining corpora lutea were seen next to moderately staining ones (Pl 2, fig 9). In the oldest animals, corpora lutea were seen which stained very little or not at all (Pl 3, fig 15).

In general, as the animals grew older, the following changes became apparent, starting at 8 months. Whole groups of interstitial cells and, to a smaller extent, thecal cells, more or less completely lost their ability to stain with FSA (Pl 3, figs 13, 15). Next to these cells, masses of pigment became visible and gradually increased with age (Pl 3, figs 12, 14). Finally, large unstained areas with a glassy appearance were seen. These were considered to be old corpora lutea (Pl 3, fig 15). These structures sometimes showed small groups of irregularly staining globules. One of the 15-month-old 'A' mice showed a completely atrophic, non-staining ovary.

Accessory sex organs

In the male, a detailed study of the *seminal vesicles* was made at various ages. An intense reaction was present in the vacuoles of the cell apex, as described in the rat (Albert & Leblond, 1946). This apical reaction was slight to moderate at 1 month, most intense at 3 months (that is to say, when males reach sexual maturity), moderate at 11 months and very slight at 15 months. While the cell apex stained intensely, the reaction in the base was only moderate. A slight reaction was present in the smooth muscle and a slight to moderate reaction in the lamina propria, probably due to the presence of elastic fibres. The secretion present in the lumen did not react.

In the female, the *uterus* showed a slight reaction in the smooth muscle and a fairly intense reaction in the epithelial cells, but only in sexually mature animals. Thus the 3-month-old 'C57' females showed a definite reaction in the epithelium and glands (Pl 2, fig 11), the apical and particularly the basal regions of the cells containing minute, brightly staining granules. In contrast, the uteri of the more slowly maturing 'A' females showed little or no reaction at 3 months (Pl 2, fig 10). Older 'A' strain females showed stained granules in the epithelium. It may be noted that scattered, intensely staining cells could be found in the mesometrium (Pl 2, fig 11). Finally,

brownish pigment was found to accumulate in the lamina propria and myometrium of the older animals

The *mammary glands* showed irregularly occurring reactions in the cells of the epithelium, apparently located on fatty inclusions (Pl 1, fig 3) Brightly staining fat globules were found free in the lumen as described by Dempsey, Bunting & Wislocki (1947)

A series of females of the high-cancer 'C3H' strain were examined with regard to the FSA reaction of cancerous and non-cancerous mammary tissue While a moderate to intense reaction was observed in the non-cancerous mammary epithelium (Pl 1, fig 3), little or no reaction was present in a cystadenocarcinoma and in three adenocarcinomata (Pl 1, fig 4)

Other organs

The reactions in the other organs were on the whole quite comparable with, although more intense than, those described in the rat (Albert & Leblond, 1946) Thus a definite reaction—slight to moderate—was present in the lymphatic organs of the mouse

The *liver* of animals of all ages showed a fairly intense staining, restricted to the cell cytoplasm The most pronounced reaction was observed in the youngest animals with a tendency towards a slow decrease with ageing Individual variations were considerable in this organ Deep-staining globules were occasionally seen in the Kupffer cells, a fact in keeping with the frequent occurrence of intense reactions in cells of the reticulo-endothelial system in all locations (Albert & Leblond, 1946)

In the *kidney* of adult animals, the reaction was maximal in the proximal convoluted tubules, moderate or fairly intense in the outer medulla (distal convoluted tubules and Henle's loops), and absent in the collecting tubules, while the glomeruli showed little or no reaction The structures showing the greatest variation with age were the proximal convoluted tubules In the newborn, the proximal convoluted tubules developing in the outer part of the organ did not react In the middle region, however, where a few differentiated proximal convoluted tubules were apparent, slight to moderate reactions were found In the innermost part of the organ, where only collecting tubules are located, no staining occurred (Pl 4, fig 18) An intense uniform staining of the proximal convoluted tubules in the cortex was observed in 1- and 3-month-old animals (Pl 4, fig 19) As the animals aged, some of the proximal convoluted tubules did not take up the stain, and eventually most of them remained unstained (Pl 4, fig 20) The occurrence of scattered, irregular globules in the collecting tubules may also be observed, especially in the older age groups

In *cardiac muscle* the reaction was found to be moderate in the newborn animals, but fairly intense in all other groups

RESULTS OBTAINED WITH FRESH (UNFIXED) TISSUES

Fresh tissues taken to FSA without passing through mercuric chloride did not stain at all On the other hand, pre-treatment with the mercuric salt resulted in an intense purple staining in most organs The reactions thus obtained were usually similar to those observed in formalin-fixed tissues Thus, the ovary showed a fairly intense reaction in the corpora lutea, thecal and interstitial cells, but not in the granulosa

The kidney showed a fairly intense reaction in most proximal convoluted tubules, and little or no reaction in the glomeruli. The outer medulla reacted in a regular fashion since all tubes showed a fairly intense staining.

Although Hayes (1947) noted that in most tissues of the rat the staining was the same before and after fixation, he emphasized the difference in the reactions of adrenal cortex and liver before and after fixation. In the mouse, however, the adrenal cortex showed the same type of reaction in the fresh state (Pl 1, fig 5) as after fixation (Pl 1, fig 1, Pl 2, figs 6, 7). The medulla, on the other hand, reacted in the fresh state (Pl 1, fig 5) but not after fixation (Pl 1, fig 1, Pl 2, figs 6, 7). The case of the liver was quite different, since only a very slight or no reaction was observed in the fresh state, while a definite staining occurred after formalin fixation.

DISCUSSION

Chemical nature of the tissue compounds reacting with FSA and DNPH

It must first be emphasized that no staining with FSA or DNPH was observed in any fresh mouse tissue without pre-treatment with mercuric chloride. Thus, there was no significant amount of free reactive carbonyls in any organ.

The intense staining observed after passage of unfixed sections through mercuric chloride—a reagent known to free carbonyls from acetal linkage—indicated that the carbonyls stained with FSA or DNPH must have been bound in this type of linkage.

Furthermore, the reacting carbonyls or their precursors could readily be extracted from the sections by fat solvents, but not by water, a fact previously noted by Hayes (1947). The lipidic nature of the reacting material suggested by this observation was confirmed by the frequent localization of the purple stain on fat droplets.

Two types of carbonyl lipids are known to exist in tissues: the ketosteroids, in which the carbonyl radical is a free ketonic group, and the plasmalogens, which are phospholipid derivatives with an aldehydic carbonyl combined in acetal linkage. From the outset, it was apparent that the plasmalogen aldehydes behaved like the carbonyls detected in sections with FSA. However, the claim of the Harvard school that FSA may be used for the histochemical detection of steroids (Dempsey, Bunting & Wislocki, 1947) makes it necessary to re-examine the possibility that ketosteroids play a role in this staining.

If ketosteroids were the reacting material, fresh tissues would stain without mercuric chloride treatment. Furthermore, the rapid development of the reaction with FSA after action of the mercuric salt indicated that the freed carbonyls must have been aldehydic rather than ketonic, since the reactivity of aldehydes was found to be considerably greater than that of most ketones as judged by sulphite addition (Petrenko-Kritschenko, 1905) or semi-carbazide formation (Conant & Bartlett, 1932). Finally, *in vitro* experiments (Oster & Oster, 1946, Albert & Leblond, 1946, Hayes, 1947, Boscott, Mandl, Danielli & Shoppee, 1948) showed that tissue extracts treated with mercuric chloride reacted with FSA in an intense fashion, while many ketones and especially ketonic steroids did not. Oster & Oster (1946) noted that some short-chain ketones reacted with FSA, namely, acetone, methyl ethyl ketone, methyl iso-butyl ketone, but they developed a red colour quite different from the characteristic purple colour obtained with aldehydes. Furthermore, the hormonally

active ketosteroids, testosterone propionate, methyl testosterone, dehydroandrosterone acetate, androstenedione and pregnenolone, gave no reaction with FSA. It was, therefore, concluded that ketosteroids were not responsible for the staining of tissues with this reagent.

The only other lipidic carbonyls known to exist in tissues were the aldehydes of the higher fatty acids, especially palmitaldehyde and stearaldehyde, which have been isolated from muscle and brain by splitting the acetal-phosphatide plasmalogen (Feulgen, Imhauser & Behrens, 1929, Anchel & Waelsch, 1942). Since the properties of plasmalogen satisfactorily accounted for those of the material detected in fresh sections with FSA, it was concluded that this material was plasmalogen. Recently, it was suggested that there existed in the body an acetal lipid lacking phosphorus and the colamine base (Newman, 1944) as well as other combined lipid aldehydes (Oster & Schlossman, 1946), but no more than presumptive evidence of their presence was given.

Incidentally, cellular components 50–200 $m\mu$ in size obtained by ultra-centrifugation of tissues gave a positive reaction with FSA. Furthermore, half the material in these particles was shown by phosphorus and nitrogen analysis to be phospholipidic (Claude, 1940, 1941). These results were thus in agreement with the opinion that the FSA-staining material in tissues consists of acetal phosphatides.

While this conclusion was based on results obtained with fresh tissues, it did not necessarily apply to fixed tissues. Thus, Hayes (1947) assumed that plasmalogen was detected by FSA after mercuric chloride treatment but only when fresh tissues were used. The present experiments, however, showed that, at least in the mouse, the localization of the reactions in most organs (ovary, adrenal cortex, kidney, etc.) was identical under fresh and fixed conditions. Therefore, the purple staining observed in fixed sections must have been derived from the same source as in fresh tissues. Plasmalogen thus appeared as being also the cause of the reactions observed in fixed tissues.

The staining of the fresh adrenal medulla (Pl 1, fig 5) contrasted with the absence of a reaction after fixation (Pl 1, fig 1, Pl 2, figs 6, 7). Probably the reacting aldehyde was extracted during the technical procedure and must therefore have been more soluble than the material in other organs and tissues. Whether the bound aldehyde present in the adrenal medulla differed from plasmalogen could only be surmised.

The liver was the only organ in which no reaction was present in the fresh condition, but some appeared after fixation. Hayes (1947) assumed that such behaviour indicated the formation of oxidation products independent of plasmalogen. However, we were able to demonstrate the presence in the liver of a plasmalogen type of compound, since a chloroform extract of this organ reacted with FSA after mercuric chloride treatment (unpublished experiments). Thus, it appeared more likely that some inhibitory mechanism prevented the reaction of plasmalogen in fresh liver sections.

The conclusion that plasmalogen was the source of the carbonyls detected in fixed tissues by FSA and DNPH must be reconciled with the result of the experiments on formalin fixation. In describing the technique, it was pointed out that the reaction was inhibited by formalin fixation, but could be reconstituted by oxidation. The possibility existed that during the process of formalin fixation, the chemistry of which

is poorly understood, some of the acetal phosphatides were slowly disrupted and the released fatty aldehydes were partly reduced. During subsequent oxidation the aldehydes would be reconstituted into an active form. That such a process occurred to some extent was suggested by the fact that reactions could take place without mercuric chloride in fixed sections, but were usually intensified by this reagent, while their localization was not altered.

It was thus concluded that, with a few possible exceptions, the reactions obtained in fresh and formalin-fixed tissues of the mouse with FSA and DNPH were due to lipid aldehydes bound as plasmalogen.

Influence of age on FSA and DNPH reactions

It is apparent that the staining of most organs with FSA and DNPH was markedly influenced by the age of the animals. In most organs, there was a gradual overall decrease of the staining intensity with advancing age. The results were most striking in the gonads and accessory sex organs. Here the reactions were slight or absent at birth, increased rapidly to reach a maximum at sexual maturity, remaining definite for a number of months during the active sexual life, and then decreasing with advancing age to become slight or to disappear in senility. Thus, for instance, in the epithelium of the seminal vesicle, the reaction became intense at puberty and disappeared in senile animals. This cycle may be compared to what occurred after castration and substitution therapy, since castration almost completely suppressed the reactions of seminal vesicles and prostate, while testosterone treatment restored them (Albert & Leblond, 1946). Therefore, the staining intensity of the seminal vesicle was dependent on its functional activity.

Similarly, there was an obvious parallel in the testis between the function of the interstitial cells and their staining reaction, which was moderate at birth (Pl 1, fig 21), most intense at puberty (Pl 4, fig 22) and slight in old age (Pl 4, fig 23). A relation between the depth of the staining and physiological activity was also encountered in the ovary (Pl 2, figs 8, 9, Pl 3, figs 12-15), in the kidney (Pl 4, figs 18-20), and to a lesser extent in the adrenal. A similar correlation between increased FSA reactions and functional activity was postulated by Tonutti (1941) to explain the intensified reactions found in the adrenal cortex of animals treated with diphtheria toxin and corticotrophin. In the skin, only the deeper, more active layers of the epidermis stained with FSA (Voss, 1941).

In unpublished experiments we found that hypophysectomy produced some decrease in the intensity of the staining reaction in the adrenals, gonads and accessory sex organs. The decrease and shift in the FSA reaction of the adrenals produced by this operation has already been examined by Deane & Greep (1946). Since removal of the hypophysis reduces the elaboration and release of steroid hormones by adrenals and gonads, these results supplied another example where staining varied as the physiological activity.

From these numerous examples it may be concluded that in many tissues the intensity of the reactions paralleled the functional state and, therefore, the amount of aldehydes combined in acetal bondage increased as the organs became actively functioning.

Since the amount of a substance present in an organ is the result of a balance

between the quantity elaborated or incorporated into the organ, and the quantity used up or excreted, the presence of a greater amount of bound aldehydes in active than in resting tissues indicated a predominance of accumulation over elimination. It is not known whether this accumulation is the result of the binding of aldehydes for their detoxication as suggested by Feulgen or is rather a preparatory step for an enzymatic release of aldehydes to be used in cellular reactions.

While the biological role of these bound aldehydes is unknown, a few preliminary conclusions may be drawn. The presence of a constant intensity of staining as in the interstitial cells of the testis should indicate a constant metabolism of plasmalogen and presumably a steady rate of secretion by all the cells, while a variable staining as in the interstitial cells of the ovary may be referable to a cyclic secretory activity.

The decrease in carbonyl staining occurring in ageing animals was frequently accompanied by an increase in the amount of brownish 'wear and tear' pigment, especially in the steroid-secreting cells (adrenal, cortex, interstitial cells of testis, thecal and interstitial cells of ovary, corpus luteum), where large, irregular cells laden with brown pigment often appeared and gradually increased in amount (Pl. 3, figs 12-15). A similar pigmentation has been described in the interstitial spaces of the testis of old mice by Hooker and Pfeiffer (1942). The steps of this pigment degeneration seemed to be as follows. At first, minute brown masses appeared in the cytoplasm of some of the cells. Later, these cells were phagocytosed by reticulo-endothelial cells, which then made up the clumps of irregular, pigment-laden masses described above. At any rate, this pigment degeneration of the steroid-secreting cells always occurred as their content in bound aldehyde decreased and their secretory role was reduced or terminated.

There is an exception to the rule that the staining with FSA and DNPH indicates the functional activity of steroid-secreting and other organs. A marked staining was observed in a few cells with signs of fatty degeneration. Thus, intensely staining fatty globules could be observed in degenerating follicles and old corpora lutea of the ovary, in the reticular zone of the adrenal, occasionally in the collecting tubules of the kidney and fairly often in the cells of the reticulo-endothelial system. In all these cases, the presence of the material in large, distended fat globules pointing to fatty degeneration cannot be taken as indicating functional activity, but rather a passive storage of the material.

More experience may be needed to assess accurately the value of FSA staining as an index of function. However, since it is most difficult to estimate the secretory ability of cells such as the interstitial cells of testis and ovary on ordinary histological slides, the technique described above may provide a new instrument.

SUMMARY

The carbonyl reagents (fuchsin sulphurous acid and dinitrophenylhydrazine) were used as histochemical stains in a study of the tissues of 'A' and 'C57' mice taken at birth and at 1, 2-3, 8-9, 11, 15 and 16 months of age. The carbonyls detected in fresh and fixed tissues appear to be aldehydic lipids held in acetal linkage, presumably as 'plasmalogen'.

The histochemical reactions obtained with the two stains have the same distribution. They are present in almost all organs and tissues, but they are most intense

in the steroid-secreting cells adrenal cortex, interstitial cells of testis and ovary, corpus luteum

The staining intensity of kidney, endocrine glands, sex glands and accessory sex organs increases until puberty, is maintained during sexual maturity and subsequently decreases with increasing age. Frequently, the decrease or disappearance of the carbonyl reactions is accompanied by pigment degeneration, especially in the steroid-secreting cells.

The parallel existing between the staining with carbonyl reagents and the physiological level of activity suggests the use of these as tests of function. Some caution is, however, necessary in the interpretation of the results.

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EXPLANATION OF PLATES

PLATE 1

- Fig 1 Adrenal of 3 month old 'A' mouse FSA stain The grey to black colour represents the purple seen in tissue sections The black spots in the medulla are usually due to blood in the blood vessels The glomerular zone stains slightly, the fascicular zone intensely, the wide reticular zone slightly, and the medullary zone not at all The darkly staining circles at the reticulo medullary junction represent the signet-ring cells described in the text Photograph taken with daylight filter on panchromatic plate $\times 40$
- Fig 2 Same organ as in fig 1, stained with DNPH and showing the same pattern of staining as with FSA Photograph taken with a purple filter on panchromatic plate $\times 40$
- Fig 3 FSA reactions in normal mammary gland of a 9 month old female 'C3H' mouse The cells of the alveoli contain intensely staining, irregular droplets, while the ducts and stroma stain slightly or not at all Photograph taken with a daylight filter on contrast plate $\times 40$
- Fig 4 Adenocarcinoma from a 9 month old female 'C3H' mouse The epithelial and stromal elements stain slightly or not at all Photograph taken as in Fig 3 $\times 40$
- Fig 5 Adrenal gland of a mouse sectioned while fresh, and stained with FSA after mercuric chloride treatment Intense reaction in both cortex and medulla Compare with sections of fixed adrenal glands (figs 1, 6 and 7) $\times 94$

PLATE 2

- Comparison of FSA reactions in organs of a 3 month old 'A' female mouse (left) and a 3 month old 'C57' female mouse (right) Photographs taken with daylight filter on panchromatic plates The black colour represents the purple reaction $\times 40$
- Fig 6 'A' adrenal The glomerular zone stains slightly, the fascicular zone intensely the wide reticular zone slightly, and the medullary zone not at all The darkly staining circles at the reticulo medullary junction represent the signet-ring cells described in the text
- Fig 7 'C57' adrenal The glomerular zone stains slightly, the fascicular zone intensely, the narrow reticular zone slightly, and the medullary zone not at all Note the absence of the darkly staining signet-ring cells at the reticulo medullary junction, and the narrowing of the reticular zone compared with fig 6
- Fig 8 'A' ovary The interstitial and thecal cells stain intensely, the granulosa cells slightly or not at all Corpora lutea are absent
- Fig 9 'C57' ovary The interstitial and thecal cells stain moderately to intensely the corpora lutea slightly to intensely, and the granulosa cells slightly or not at all The presence of corpora lutea indicated an early maturity in this strain
- Fig 10 'A' uterus The immature muscularis and epithelium stain slightly or not at all
- Fig 11 'C57' uterus The well developed muscularis stains slightly or not at all, the basal portions of the epithelial cells intensely, and the apex only slightly A concentration of positively staining material may be seen in the mesometrium

PLATE 3

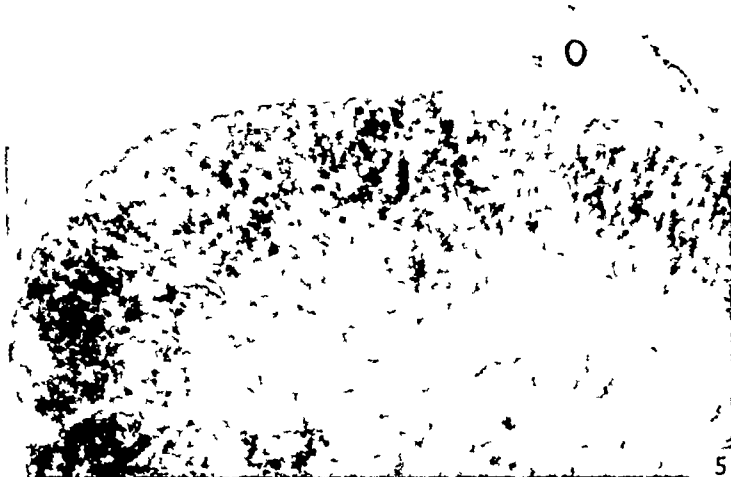
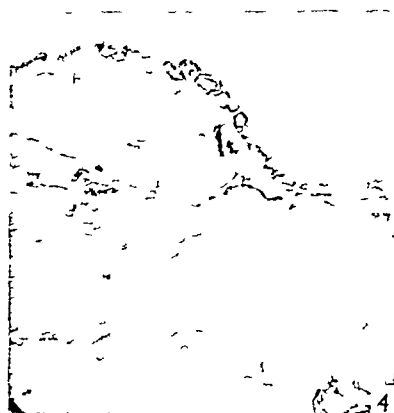
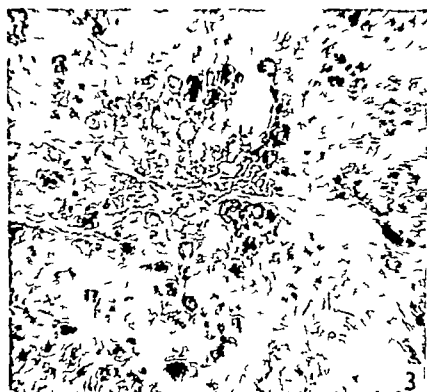
- FSA reactions in senile animals Figs 12 and 14 were taken with a daylight filter on contrast plates and show in black both the purple staining reaction and the brown pigment Figs 13 and 15 were taken with a green filter on panchromatic plates to show in black the purple reaction only It is therefore possible to deduce the location of the brown pigment, which is the material appearing in black in 12 and 14 but not in 13 and 15 $\times 40$

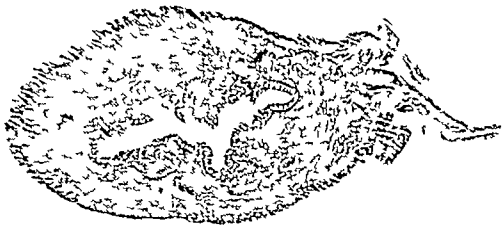
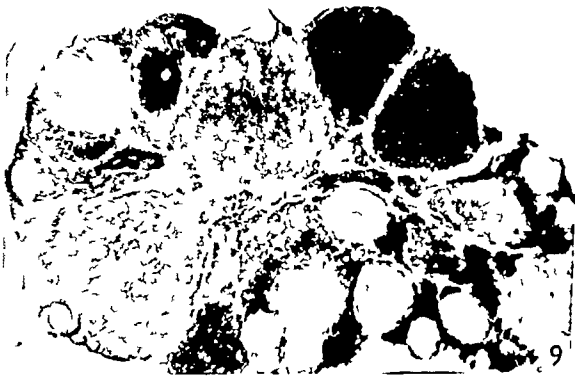
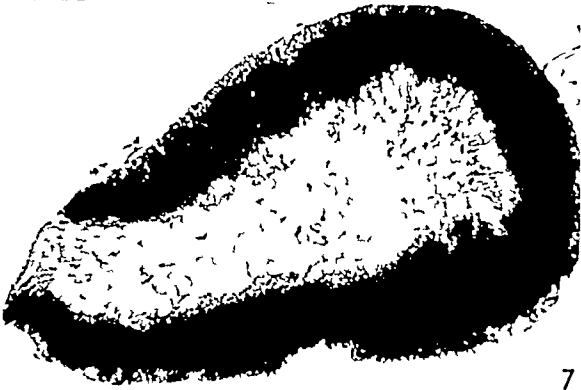
- Fig 12 8 month old 'C57' mouse ovary The deposition of the non staining but light absorbing brown pigment in the interstitial cells can be determined by the presence of black interstitial masses visible in this and not in the next figure
- Fig 13 Same tissue as fig 12 showing in black the FSA stain only Only one corpus luteum and rare groups of thecal and interstitial cells stain
- Fig 14 16 month old 'A' mouse ovary The brown pigment can be recognized by the dark spots in this figure
- Fig 15 Same tissue as fig 14 showing only FSA stain The entire ovary, with the exception of the cells lining an ovarian cyst, stains slightly or not at all The hyalinized corpora lutea do not stain at all
- Fig 16 16 month old female 'A' mouse adrenal The glomerular zone stains moderately, the outer half of the fascicular zone moderately, and the inner half intensely Abundant aggregations of darkly staining cells extend from the inner fascicular zone into the indistinct reticular zone A small amount of pigment is also present
- Fig 17 16 month old female 'A' mouse adrenal The glomerular zone stains slightly, the outer half of the fascicular zone slightly to moderately, and the inner half intensely, the reticular zone slightly, and the medulla not at all Throughout the reticular zone are scattered intensely staining aggregations of cells

PLATE 4

Variations of FSA reaction in kidney (left) and testis (right) with age The black colour represents the purple seen in the tissues Photographs taken with a daylight filter on contrast plates $\times 40$

- Fig 18 1 day old mouse kidney A few differentiated proximal tubules in the middle region stain slightly No stain anywhere else
- Fig 19 3 month old mouse kidney Fairly intense reaction in the proximal convoluted tubules
- Fig 20 16 month old mouse kidney Slight or no staining throughout the organ This kidney showed the least intense reaction of all those examined
- Fig 21 1 day old mouse testis The interstitial cells stain fairly intensely and the poorly developed spermatogenic tubules slightly or not at all
- Fig 22 3 month old mouse testis The interstitial cells stain intensely and the spermatogenic tubules slightly or not at all, with a few dark staining granules about the heads of the spermataids
- Fig 23 15 month old mouse testis The atrophied cells of the interstitial spaces and the spermatogenic tubules stain slightly or not at all







12



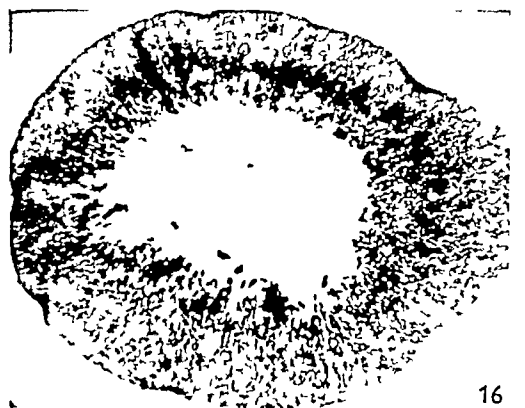
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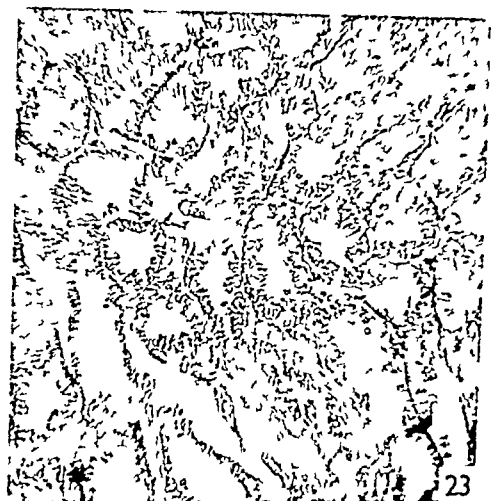
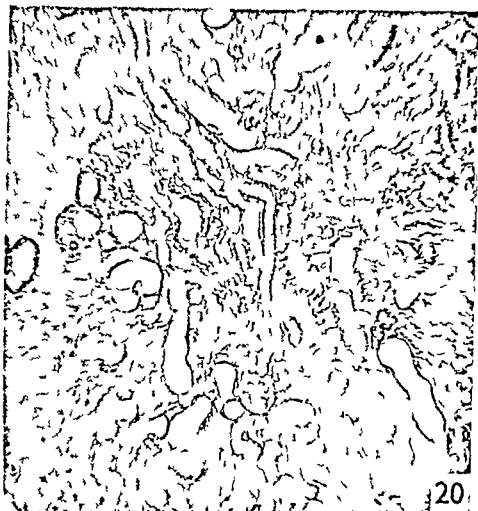
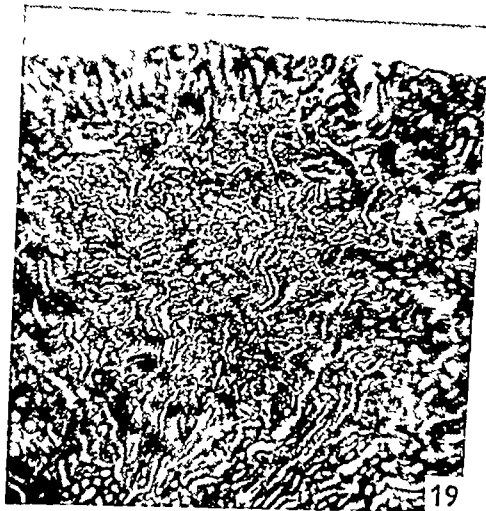
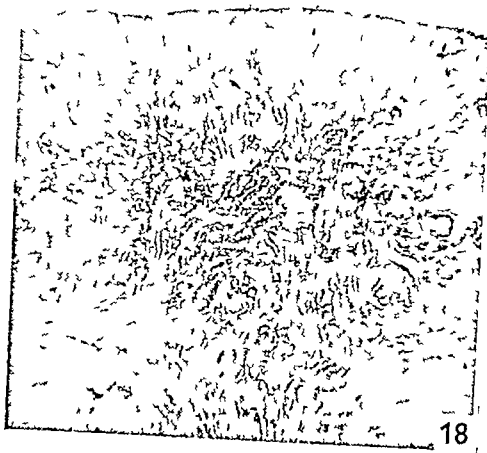
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17



A COMPARISON OF ENDOCRANIAL CAST AND BRAIN OF AN AUSTRALIAN ABORIGINE

By A. D. PACKER, *Department of Anatomy, University of Adelaide*

A comparison of an endocranial cast with the corresponding brain is the obvious method of determining how much accurate information about the brain can be obtained from the cast. Symington (1915) using human European material made such a comparison. His studies showed that very little of the detail of the sulcal pattern was evident on the cast—he was able to identify only the lateral sulcus and its posterior branch, the superior and middle temporal sulci and the sulci related to the orbital surface of the frontal bone. He also showed that the sulcal pattern, especially of the vault, was obscured by the structures lying between brain and skull, viz meninges, arachnoid granulations, cerebrospinal fluid, cerebral veins and lacunae, venous sinuses and meningeal vessels. On the basis of these investigations he criticized (1916) the interpretations of a number of workers on casts from fossil skulls.

Le Gros Clark, Cooper & Zuckerman (1936) made a similar study in the chimpanzee and concluded that little information about the convolutional pattern of the brain could be derived from the cast—they were able to identify with certainty the rostrum of the lateral sulcus, the fronto-orbital sulcus, the superior and inferior frontal sulci and the inferior pre-central sulcus, vaguely or inconstantly marked were the lunate sulcus, the central sulcus, the parallel sulcus, the angular sulcus and the inferior temporal sulcus.

Both of the above-mentioned investigations showed that there were depressions on the casts not corresponding to the positions of sulci, indicating that the mapping of sulci on an endocranial cast without the brain for comparison would be liable to error.

The endocranial cast of the Australian aborigine has been used in morphological studies, mainly for comparison with other casts, by Keith (1925), Elliot Smith (1928), Kappers (1929), Shellshear & Elliot Smith (1934) and Hirschler (1942). Shellshear (1934) described the cranio-cerebral relations of one cerebral hemisphere. No direct comparison of cast with corresponding brain has been published. Therefore, it seemed of value to make such a comparison, in order partly to bridge the gap between Symington's findings for the European and those of Le Gros Clark *et al* for the chimpanzee.

MATERIAL AND METHODS

The brain and endocranial cast were from a full-blooded male Australian aborigine aged 64 years. The body had been injected with a solution containing 5 % formalin. Nine days after the injection the skull-cap was sawn through and removed, and the brain removed *in toto*, the brain stem being cut at the level of the foramen magnum. The brain was in good condition with no distortion and no obvious shrinkage, photographs and measurements were made on the day of removal, before any drying could occur.

The dura was stripped as cleanly as possible from the whole of the inside of the skull—some difficulty was encountered in the region of foramina—and a cast was prepared, using melted printer’s gelatin. A hole (20 mm diameter) was bored through the skull near the vertex, the skull-cap was firmly fixed in position and the gelatin poured in through the hole. From the gelatin cast a plaster mould was prepared and finally a plaster cast, using fine dental plaster of Paris in both procedures. The volume of the cast was estimated and measurements taken. Hirschler’s base-line joining the hindmost point of the cast to the most medial point of the marking of the fronto-marginal sulcus (B N A) was employed. Using an oblique beam of light the depressions on the cast were carefully marked in pencil, this was done without reference to brain or skull. Tracings of photographs of various views of brain and endocranial cast were then superimposed (with correction for slight differences in size) and the general contours were compared, any sulci corresponding to depressions on the cast were noted, particular attention being paid to the central, lateral and lunate sulci. Obvious eminences on the cast were outlined and compared with corresponding regions of the brain.

The terminology of the brain sulci is in the main that used by Shellshear (1937) in his study of brain morphology of the Australian aborigine.

MEASUREMENTS

The measurements made on brain and cast are given in the following Table. The brain stem was cut in the region of the foramen magnum and the cerebral peduncles were divided at their junction with the pons. The weight includes pia-arachnoid and blood vessels.

	Brain		Cast
Weight	Forebrain and midbrain	920 g	
	Hindbrain	171 g	
	Total	1091 g	
Volume (water displacement)	Forebrain and midbrain	1010 c c	1270 c c
	Hindbrain	142 c c	
	Total	1152 c c	
Maximum length	Left side	166 mm	Left side 173 mm
	Right side	160 mm	Right side 166 mm
		112 mm	117 mm
Maximum breadth			
Base line (Hirschler)	Left side	160 mm	Left side 166 mm
	Right side	155 mm	Right side 162 mm
Height index (Hirschler*)	Left side	38.1	Left side 45.1
	Right side	37.2	Right side 41.0
Depth index (Hirschler*)	Left side	21.9	Left side 18.1
	Right side	22.8	Right side 18.4

* Hirschler’s height index is the greatest height of the brain above the base line, expressed as a percentage of the base line. His depth index is the maximum depth of the temporal lobe below the base line expressed as a percentage of the base line.

Duckworth (1903) found the average capacity of 150 Australian aboriginal skulls to be 1246 c c. Davis (1869) estimated the average weight of 17 male brains to be 1197 g. Thus the cast here studied is above average volume but the brain is less than average weight.

GENERAL CONTOUR

A brief description of the contour of the cast is given and mention made of the regions in which the cast does not give an accurate indication of the corresponding surface of the brain

Superior midline region (Figs 1-4, 7 and 8, throughout, the odd-numbered figures are of the cast and the even-numbered of the brain)

The frontal lobes of the cast slope upwards from the rounded symmetrical poles with an even convexity (comparable with that of the brain) as far as a centrally placed bregmatic eminence, which is an obvious midline elevation with its centre approximately at the bregma and measuring 45 mm long by 40 mm wide—this eminence does not correspond to any elevation of the brain, which is rather flattened in this region. It almost certainly marks the position of the bregmatic 'pool' of cerebrospinal fluid. Arachnoid granulations cause irregularities on its surface.

Immediately behind this 'pool' the contour of the midline is obscured by the hole bored in the skull to make the cast. Just posterior to this region and on either side of the midline are two oval longitudinal elevations, not well defined at their periphery and not symmetrical, their surfaces are made irregular by the markings of several arachnoid granulations. On the left side the approximate size is 50 by 30 mm, on the right the elevation is slightly longer and rather narrower. The brain in this region shows no definite elevations, although it is at the maximum convexity of curvature at the upper part of the occipital lobes. The elevations are probably due to 'parietal pools' of cerebrospinal fluid.

Behind these eminences the surface of the cast is flattened, but there is no definite depression. The curvature of the corresponding brain surface is regular and rounded, suggesting that the area of flattening on the cast is determined mainly by the presence of the 'parietal pool' eminences lying in front of it.

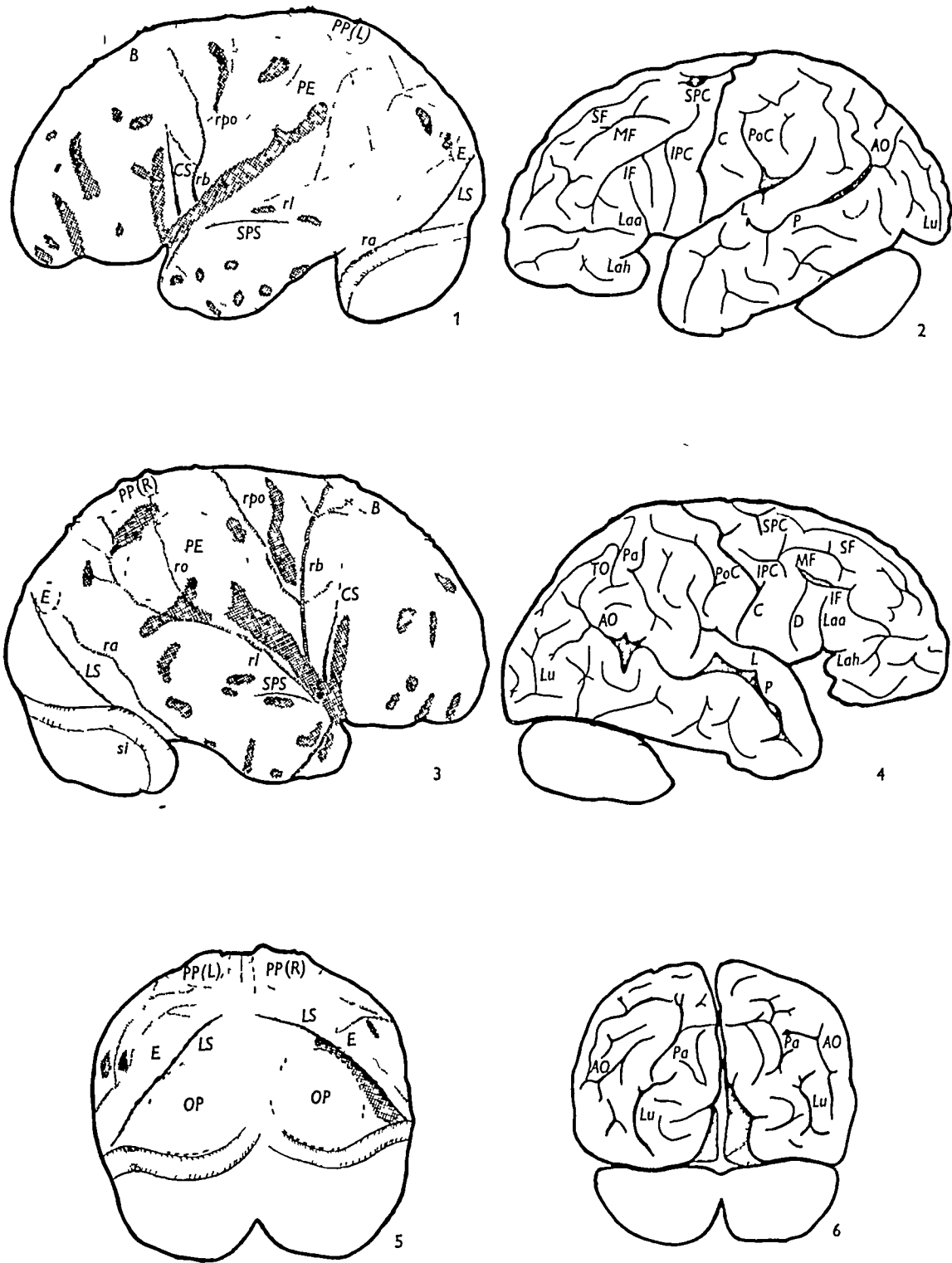
The occipital poles are very prominent on the cast, they are asymmetrical, the left being more pointed and projecting more posteriorly, both poles lie well behind the lambdoid suture markings and overlap the cast of the cerebellum by about 10 mm. There is a comparable asymmetry and overlap in the case of the brain.

Lateral regions (Figs 1-8, 11 and 12)

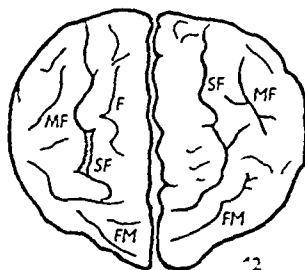
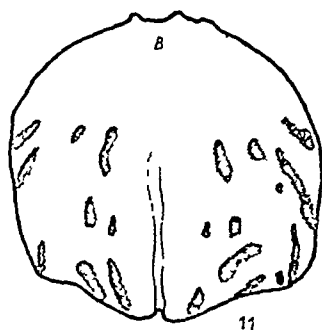
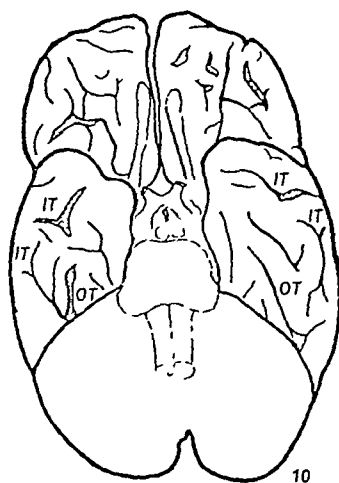
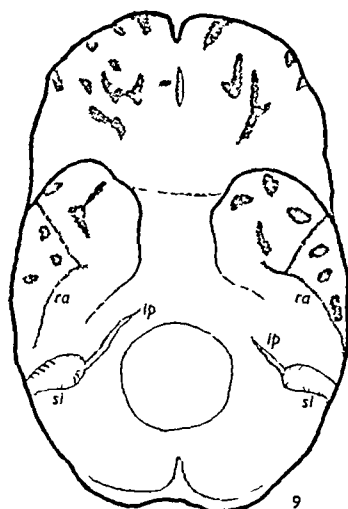
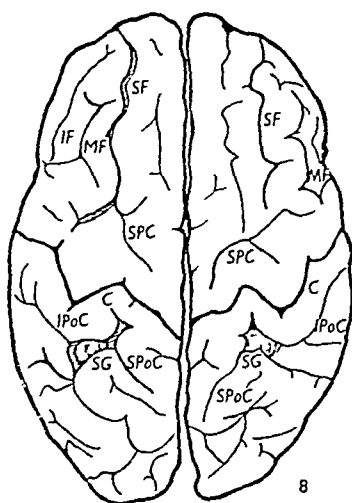
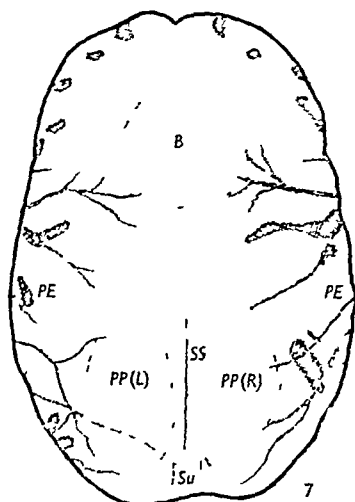
The most obvious features of the cast are the parietal eminences (outlined in Figs 1 and 3), they are definite elevations on both sides lying just above and behind the posterior limb of the lateral sulcus, at the periphery the eminences are ill-defined, but their shape is on the whole circular. There are similar but less obvious elevations of the brain formed by the lower part of the post-central gyrus and the inferior parietal lobule. The parietal eminence on the right side of the cast is slightly more prominent than on the left, and this difference is present in the brain also.

Also noticeable is the tendency to constriction of the cast immediately in front of the coronal suture markings, the brain is constricted to a similar degree.

Situated just in front of the markings of the lambdoid sutures and approximately 35 mm lateral to the lambda are two small rounded elevations of diameter less than



Figs 1-6 For explanation see end of article



Figs 7-12 For explanation see end of article

20 mm They are symmetrical in position, the right is slightly more obvious than the left, but neither is well defined There are no corresponding elevations on the brain

The contours of cast and brain over the remainder of the lateral regions correspond fairly accurately and need no further description

Frontal and inferior regions (Figs 1-4 and 9-12)

Near the midline the rounded frontal poles of the cast continue inferiorly into a rather blunt orbital keel which projects downwards for only a short distance Compared with the brain the keel is slightly broader and projects downwards a little more, but it represents quite closely the brain contour

The orbital surfaces of the cast are concave from side to side and less markedly so from before backwards As the medial border formed by the keel projects farther downwards than does the lateral, the orbital surfaces look laterally as well as downwards The lateral border has several notches in it and ends posteriorly in a projection marking the position of the orbital operculum, which is more obvious on the left side of both cast and brain These opercula are more prominent on the cast and in frontal view give a quadrangular outline compared with the more rounded contour of the brain

In the brain the insula is partly exposed on both sides, particularly on the right, whereas on the cast the notch at the stem of the lateral sulcus is wider on the left

The contour of the temporal lobes of the brain is accurately shown by the cast

SULCAL PATTERN

Frontal region (Figs 9-12)

On the orbital surface of the frontal lobe of the cast there is some sulcal pattern present, but it does not accurately represent the sulci of the brain—some of the widest sulci not being represented on the cast, while some small but wide depressions on the gyri are well marked on the cast On the upper and anterior surfaces of the frontal lobe there are several ill-defined depressions on the cast, on both sides, those corresponding to the superior frontal sulci can be made out fairly readily On the right side comparison with the brain shows that there is a depression on the cast corresponding to a wide longitudinal furrow on the superior frontal gyrus Two other depressions lie along the line of the fronto-marginal sulci None of the other grooves accurately corresponds to a sulcus but several of the smaller ones coincide with short wide depressions on gyri

Parietal region (Figs 1-4)

The stem of the lateral sulcus and its posterior limb are accurately indicated on both sides of the cast by a well-marked groove extending to just below the parietal eminence The anterior horizontal ramus are not indicated on either side On both sides of the cast there is a definite short groove lying just in front of the coronal suture marking the anterior ascending ramus lie mainly just anterior to these grooves although the lower part of the left anterior ascending ramus coincides with the lower part of the groove It is probably more accurate to say that the groove indicates the depressed part of the brain between the fronto-parietal and frontal opercula On

neither side of the cast is the central sulcus accurately indicated. On both sides there is a depression lying posterior to the upper part of the bregmatic branch of the middle meningeal artery and in each case the lower part of this groove coincides with the position of the middle of the central sulcus, the coincidence being more exact on the left side. The upper parts of the central sulci lie posterior to the groove. No other grooves of the parietal region accurately correspond to any sulci. There is a definite short depression just above and posterior to the right parietal eminence which corresponds with a partly depressed gyrus (the anterior part of the superior parietal lobule) lying immediately posterior to the upper end of the post-central gyrus. However, on the left side of the brain there is a similar sunken gyrus but no groove on the cast.

Occipital region (Figs 5, 6)

There is no sulcal pattern obvious in this region of the cast. On the right side there is a groove lying immediately behind and parallel to the lambdoid suture marking, but this does not correspond to any sulcus on the brain. The lunate sulci are situated entirely posterior to the lambdoid sutures. The sulcus on the left side is wide and deep compared with that on the right, but it is not represented on the cast. The lunate sulci are farther from the lambdoid suture than Shellshear (1934) found in his material.

Temporal region (Figs 1-4, 9 and 10)

In this part of the cast there are some short, relatively well-marked depressions on the lateral and inferior surfaces. They do not lie accurately along the line of the sulci on the right side, but on the left side four such small notches mark the position of the anterior end of the inferior temporal sulcus. The parallel sulci are not represented on the cast, in spite of the fact that on the right side of the brain the sulcus is widely open.

MARKINGS OF VESSELS AND CRANIAL SUTURES

These markings are very similar to those on endocranial casts of European man. They are illustrated in Figs 1, 3, 5, 7 and 9. The well-marked ridges formed by the middle meningeal vessels are named according to Ruggeri (1913), and the pattern of the ridges corresponds to type I of that author's classification for modern man. The pattern also agrees in basic plan with that depicted by Schepers (Broom & Schepers, 1946).

DISCUSSION

In general, the endocranial cast here described may be said to give a fairly accurate indication of brain shape and contour. The main discrepancies are due to provision for cerebrospinal fluid circulation, the bregmatic and the two parietal 'pools' causing definite elevations near the midline. It is probable that lacunae laterales and cerebral veins as well as arachnoid granulations take part in forming these eminences. The apparent flatness of the cast in the region between the parietal 'pools' and the occipital poles can be interpreted as due to contrast with the adjacent elevations. This interpretation is strengthened by considering the brain contour, which is more

convex than that of the cast in the region concerned. Shellshear & Elliot Smith (1934) have mentioned a lack of development in the pre-occipital region of the Australian aborigine, but it is not clearly evident in this brain. Although there is some fullness of the cast in the region of the coronal suture, there is no definite evidence of any subcoronal stream of cerebrospinal fluid as described and depicted by Keith (1931) for modern man and some lower types.

The sulcal pattern is poorly marked on the cast, this is in general agreement with the other brain/cast comparisons made in the European and the chimpanzee.

A number of short, relatively deep depressions on the cast coincide in position with widely open pits or furrows along the course of some gyri (most obvious in the temporal region), or with widely open parts of sulci (e.g. the left inferior temporal sulcus). These findings support those of Hirschler who has pointed out that widely open rather than deep depressions on the brain are more likely to correspond with depressions on the cast, and that such depressions are of little value in working out the sulcal pattern. On the other hand, a number of depressions on the cast do not appear to coincide with any hollows of the brain surface. Hirschler has also mentioned that a depressed gyrus may cause a groove on the cast, and this is shown on the right side of this cast. However, as the groove is situated between the elevation of the right parietal 'pool' and the right parietal eminence, there must be some doubt about this interpretation, the more so when one considers that on the left side of the cast there is no depression corresponding to a similar sunken gyrus. There are other instances of widely open brain depressions (e.g. the right parallel sulcus and the left lunate sulcus) not obscured by the bregmatic or parietal 'pools' and yet not represented on the cast. Obviously, the various structures and the cerebrospinal fluid lying between brain and skull must play a part as 'obscuring factors'.

The sulcal pattern of the cast is also made more difficult of interpretation by the markings of the meningeal vessels. The main vascular markings are well-defined ridges which give the impression that there is a groove on either side of them. The most obvious example of this is in relation to the marking of the central sulcus whose middle part coincides with a groove between the bregmatic branch of the middle meningeal vessels and the parietal eminence. The lower part of this groove does correspond in position to the middle of the central sulcus, but whether there is a direct relationship seems more doubtful.

Hirschler and others have indicated the difficulty of deciding from a cast the degree of exposure of the insula. This is well borne out here, for, on the cast, the stem of the lateral sulcus is wider on the left side, while, on the brain, the insula (because of the poorly developed orbital and frontal opercula) is more exposed on the right. The explanation of this discrepancy is not apparent.

The brain/endocranial cast studies on primates so far made all show the difficulty of correctly inferring the sulcal pattern from the cast markings, even in those regions where there are well-marked grooves. It seems probable, therefore, that the interpretation of the sulcal pattern on endocranial casts from fossil skulls is likewise liable to error. It is suggested that such casts might well be restudied after an extensive preliminary comparison of brain and endocranial cast in living primates.

SUMMARY

1 An endocranial cast prepared from the skull of a full-blooded Australian aborigine is compared with the corresponding brain

2 The shape and contour of brain and cast correspond fairly accurately except superiorly, near the midline, where elevations on the cast are produced by collections of cerebrospinal fluid, arachnoid granulations and probably, also, by lacunae laterales and cerebral veins

3 The sulcal pattern is poorly marked on the cast and few sulci are easily identified, a number of the grooves on the cast do not coincide with the position of sulci. Some of these grooves indicate the situation of short wide depressions on the brain, usually along the course of gyri, but sometimes along sulci

4 On the other hand, several wide sulci or parts of sulci are not represented on the cast

5 The markings on the cast caused by vessels and sutures are illustrated. These markings (and the midline elevations) tend to give an erroneous idea of the presence of grooves lying alongside them

6 The degree of exposure of the insula cannot be inferred from the cast

7 These results are in general agreement with those of other more recent brain/endocranial cast comparisons in modern man and the chimpanzee

This work was commenced in 1939 at the suggestion of Prof. F. Goldby, to whom I am indebted for advice and references. It was completed in 1948 with the encouragement of Prof. A. A. Abbie, who gave further advice and further references to literature.

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EXPLANATION OF FIGS 1-12

All drawings are reduced to just less than half size

Figs 1, 3, 5, 7, 9, 11 are tracings from photographs of different views of the endocranial cast. Depressions are indicated by cross hatching, elevations, vascular and sutural markings are also shown.

Figs 2, 4, 6, 8, 10, 12 are tracings from photographs of comparable views of the brain. Widely open sulci are indicated by shading, the sunken gyri are also shown. The main sulci are labelled according to Shellshear (1937).

Abbreviations

<i>AO</i>	anterior occipital sulcus	<i>OT</i>	occipito temporal sulcus
<i>B</i>	bregmatic 'pool' eminence	<i>P</i>	parallel sulcus
<i>C</i>	central sulcus	<i>Pa</i>	paroccipital sulcus
<i>CS</i>	coronal suture	<i>PE</i>	parietal eminence
<i>D</i>	diagonal sulcus	<i>PoC</i>	post central sulcus
<i>E</i>	small eminence above lambdoid suture	<i>PP (L)</i>	left parietal 'pool' eminence
<i>F</i>	longitudinal furrow on superior frontal gyrus	<i>PP (R)</i>	right parietal 'pool' eminence
<i>FM</i>	fronto marginal sulcus	<i>ra</i>	ramus astericus
<i>IF</i>	inferior frontal sulcus	<i>rb</i>	ramus bregmaticus
<i>ip</i>	inferior petrosal sinus	<i>rl</i>	ramus lambdaticus
<i>IPC</i>	inferior pre central sulcus	<i>rpo</i>	ramus pre obelicus
<i>IPoC</i>	inferior post-central sulcus	<i>ro</i>	ramus obelicus
<i>IT</i>	inferior temporal sulcus	<i>SF</i>	superior frontal sulcus
<i>L</i>	lateral sulcus	<i>SG</i>	sunken gyrus (anterior part of superior parietal lobule)
<i>Laa</i>	anterior ascending branch of lateral sulcus	<i>si</i>	sigmoid sinus
<i>Lah</i>	anterior horizontal branch of lateral sulcus	<i>SPC</i>	superior pre central sulcus
<i>LS</i>	lambdoid suture	<i>SPoC</i>	superior post central sulcus
<i>Lu</i>	lunate sulcus	<i>SPS</i>	squamo parietal suture
<i>MF</i>	middle frontal sulcus	<i>SS</i>	sagittal suture
<i>OP</i>	occipital pole	<i>Su</i>	superior sagittal sinus
		<i>TO</i>	transverse occipital sulcus

BONES, MUSCLES AND VITAMIN C

II PARTIAL DEFICIENCIES OF VITAMIN C AND MID DIAPHYSEAL THICKENINGS OF THE TIBIA AND FIBULA IN GUINEA-PIGS

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INTRODUCTION AND EARLIER OBSERVATIONS

In earlier papers (Kodicek & Murray, 1943, Murray & Kodicek 1946) we briefly reported the formation, in guinea-pigs kept on diets partially deficient in vitamin C, of very extensive diaphyseal thickenings of the tibia and fibula. The present paper contains a more complete report on those changes, which were centred about the middle of the diaphyses. Other structural changes which occurred at the proximal ends of tibial diaphyses in the same material are described in the third paper of this series. The experiments were those described in the first paper of the series (Murray & Kodicek, 1949), they are summarized in Table 1 of that paper.

This is not the first occasion upon which the development of new sub-periosteal bone has been observed in guinea-pigs during prolonged partial deficiencies of vitamin C. Hojer (1924) constantly found osteoporosis of the jaw accompanied by the formation of spongy porous bone on the surface. His illustrations leave no doubt that this spongy bone resembled that described on limb bones in the present paper, indeed, Hojer comments that a similar tissue was formed on the diaphyses of long bones in the presence of a fracture or other circumstances leading to increased periosteal activity.

More recently, Mouriquand and his colleagues (1937, 1938, 1940) have described diaphyseal thickenings as seen in radiographs which leave no doubt of the identity of the phenomenon with that which we have observed, but so far as we are aware they have published no histological study. They, like us, find the thickenings to be formed in prolonged partial vitamin C deficiency but, since they made no fractures, they did not observe the connexion with injury (see below, p. 214). We have not seen the irregular osteophytes which they describe, but as these are said to depend for their formation on the presence of lemon juice in the diet which we did not use, this is not surprising. Some of their radiographs show the bow-legs and the slope of the proximal epiphysis of the tibia described below.

A number of authors (e.g. Baetjer, 1919, Bromer, 1928, 1943, Scott, 1941, Wimperger, 1935) have described the appearance of very large subperiosteal haematomas on the long bones of children suffering from scurvy (Barlow's disease). These calcify, and probably ossify, after organization. Similar changes were found in monkeys by Hart & Lessing (1913).

* This work was done while the first author was at the Department of Biology, St Bartholomew's Hospital Medical College.

Finally, we are permitted to mention a comment by Mr Last of the Royal College of Surgeons, made after seeing some of our material, that he had seen radiographs which showed diaphyseal thickenings in the legs of scorbutic Ethiopians, closely resembling ours taken from guinea-pigs. Of course, similar thickenings are well known in Man under circumstances not suggestive of any vitamin deficiency, as in the chronic periostoses of syphilis, and in obscure circumstances in infants (Caffey 1946, von Chian, 1938, Ellis, 1938-9)

EXPERIMENTS, MATERIAL AND METHODS

These were the same as for the first paper of this series (Murray & Kodicek, 1949), and are there described. The most important features of the experiments are summarized in Table 1 of that paper.

MID-DIAPHYSEAL SUBPERIOSTEAL THICKENINGS

Mid-diaphyseal thickenings in normal animals

The subperiosteal thickenings seen in the animals kept on diets partially deficient in vitamin C proved to be an exaggeration of a change seen in the tibiae and fibulae of dietetically normal animals with fractured fibulae. In these animals (group 1 of Exps 3-6), the radiographs taken at 14 or 19 days after the operation (the earliest, except those made on the day of the operation itself) showed a thickening of the tibial and fibular diaphyses, in the operated legs (Pl 1, figs 1, 2B). In the fibula this occurred in the neighbourhood of the callus, and was indeed an extension of it, but might extend for considerable distances above and below the site of the fracture, and was evidently to be identified with the 'distant periosteal reaction' described by Hertz (1936). In the tibia it appeared from the radiographs to be related to the mid-diaphyseal region, where the concavity of the bone is greatest, rather than to the position of the fracture in the fibula. It was nearly always a slight superficial thickening such as is shown in Pl 1, fig 1, but in a few cases it was larger.

The radiographs suggested that these thickenings were due to the deposition of new periosteal bone, and this was confirmed by sections (Pl 1, fig 5). The mode of their formation in dietetically normal animals was investigated in eight guinea-pigs in each of which a fibula was fractured and which were killed 1, 2, 3, 4, 5, 6, 7 and 9 days later. These animals are included in Table 1 as part of Exp 1. We studied longitudinal sections which passed through the tibia and fibula of the operated legs, including the region of the fractures and a considerable length of both bones above and below it.

No description will be given here of fracture repair in these animals, because it is hoped that one of us (P D F M) will discuss certain of its problems later. The conditions foreshadowing the development of the thickenings are seen in the animals killed on the first to third days after the fracture. In these, the periosteum of both tibia and fibula is, over wide areas, lifted away from the bone by oedema and haemorrhage beneath it (Pl 1, fig 6). In the specimens killed on the second and third days, cells had filled the space between the fibrous layer of the periosteum and the bone (Pl 1, fig 7) and mitoses were numerous among them. In the animal killed at 4 days there was very early bone formation in the enlarged cambial tissue thus

formed on the lateral surface of the tibia. The first new bone contained in its matrix great numbers of small basophil granules. These were also formed in the callus which later developed about the point of fracture, and in the subperiosteal thickenings of animals on diets partially deficient in vitamin C, they will be mentioned again in the latter connexion. At 4 days, the formation of the new subperiosteal bone had begun on the fibula (but not in the immediate neighbourhood of the fracture, where the periosteum was dead or absent, having presumably contracted away), and on the adjacent lateral surface of the tibia (Pl 2, fig 8). In succeeding days, there was further development of this bone, resulting in the formation of short thick trabeculae extending from the old surface of the diaphysis to the fibrous layer of the periosteum (Pl 1, fig 5). It is noteworthy that the new bone is not a simple addition to the old, formed by an increased rate of deposition of compact bone resembling that already forming the diaphyseal wall, but is architecturally a new structure.

In animals killed many days (e.g. 94, 102) after the operation, and kept throughout on a diet fully supplemented with the necessary vitamins, the trabecular bone thus formed was found to have been made compact by deposition on the trabeculae, the spaces between them being thus reduced to no more than vascular channels. Nevertheless, the irregular orientation of the fibrillar structure still made it easy to distinguish between the new bone and that of the original diaphysis, with its much more regular structure. Frequently, layers of regular bone, in all respects normal, had been formed externally, by deposition on the surface in a cambial layer which was no longer abnormally thickened. The bone earlier formed in the widened cambium, after injury, could then be seen sandwiched between two layers of more regular bone, one situated internally, and the other externally to it, or it might be found lining the marrow cavity if the original diaphyseal wall had been completely removed from within in the course of normal growth of the tibia.

Animals on diets deficient in vitamin D

These animals showed no differences of any kind from the normal animals.

Mid-diaphyseal thickenings in animals kept on diets partially deficient in vitamin C

In these animals the diaphyseal thickenings might be absent, and when present might range from thickenings as small as in the animals on normal diets to very large indeed (Pl 1, fig 2D, Pl 2, fig 9). Table 1 summarizes the occurrence, in the partially deficient groups of Exps 3-6, of thickenings which were larger than were usually seen in animals of groups 1 and 2 of these experiments (which had ample vitamin C throughout). The Table includes both mid-diaphyseal thickenings of the tibia and fibula, and similar thickenings at the proximal end of the diaphysis.

Table 1
(Explanation in text)

Exps 3 and 4, group 3	5 cases out of 6 legs
4	9
Exps 5 and 6, group 3	10
4	19
5	9
6	1
	8
	2
	8

The structure of the thickenings

The new bone which formed the thickenings was deposited wholly on the surface of the old. The thickenings were thus periosteal structures, and were therefore limited to the diaphyses of the bones.

In the normal bone of the diaphysis of the guinea-pig's tibia or fibula, the fibrous architecture is longitudinally arranged and shows a lamellar structure only in part. The remainder of the bone is, in the words of Weidenreich (1923, 1930), and other German authors, a parallel-fibred fine fibrous bone. The new bone is not a mere continuation and enlargement of the existing structure, but has a new and quite different architecture (Pl 2, fig 11) which is abruptly joined to the old and does not pass into it by gradual transition. Usually, one or several lines of 'cement' intervene at the periphery of the old bone and demarcate it from the new. Often, however, recognition of the boundary between the old and the new is made difficult by the extreme degree of porosis which the old may suffer, for it may be so dissected that its fragments simulate trabeculae of the new bone, and this resemblance may be increased by the deposition of narrow layers of new bone over the surfaces of the remaining parts of the old.

In transverse sections of the diaphysis of an affected bone (Pl 2, figs 9-11), the thickening usually consisted of rather narrow bony trabeculae radiating to the periosteum and joined by occasional cross connexions. Tangential sections (Pl 2, fig 12), however, showed that the radiating trabeculae were really flattened plates running longitudinally, at right angles to the surface of the old bone, from it to the periosteum. How far this verbal description exaggerates the regularity of the structure will be seen from the photographs.

Most of our sections showed the new radiating trabeculae arising from the compact but now porotic original bone of the diaphyseal wall, but in some cases the resorption of the compacta from the side of the marrow, occurring in the normal course of growth, combined with the porosis and with the superficial resorption which often precedes the development of the new trabecular bone (see below), led over wide areas to the total destruction of the old bone. The diaphyseal wall then consisted in these regions entirely of the radiating trabeculae. In other cases it could be seen that the destruction was not quite complete, an almost entirely trabecular wall being completed by a narrow zone of old compact bone (Pl 3, fig 13).

At the surface of the new bone there was often found some attempt to form a peripheral bony shell joining the ends of the radial trabeculae, this structure was usually very incomplete (Pl 3, fig 14).

The surface of the thickening was covered by the fibrous layer of the periosteum. Beneath this, there might be groups of cells clustered round the ends of trabeculae still growing in length, or covering the surfaces of the developing peripheral bony shell. Where the general connective tissue of the limb, outside the periosteum, was in the hyperplastic condition described in the earlier paper of this series, the fibrous layer of the periosteum tended also to be involved, and might in part disappear, dissolving into the cellular and fibrillar tissue which replaced the normal soft tissues of the leg. Such dissolution of the periosteum, when seen at all, was confined to part only of the circumference in transverse sections. Where it occurred, there was no sign of any attempt by the new bone to form a superficial 'shell'. The trabeculae

appeared to radiate outwards into the general connective tissue of the leg, but in fact they did not become longer in these regions than elsewhere, i.e. they did not pass beyond the zone which could be recognized, by extrapolation from the remaining parts of the periosteum, as 'belonging' to the bone.

The tissue between the trabeculae of the new bone was a loose connective tissue with a delicate fibrillar stroma whose fibrils ran into the bone. The cells had usually the appearance of fibroblasts, many of them were flattened against the trabeculae. In places cells were found against the bone in more or less regular rows, or grouped around the ends of the trabeculae. There can be no reasonable doubt that these were osteoblasts, but they often lacked the regular form and arrangement of normal osteoblasts, a feature which has often been reported in animals suffering from various degrees of scurvy.

Vascularity of the thickenings

A remarkable feature in many of the fully developed thickenings in animals which had been for long periods (up to 17 weeks) on the experimental diets was the small number of blood vessels in the connective tissue between the trabeculae. In some, there were a few channels resembling capillaries but without red corpuscles, while others seemed to be completely, or almost completely, avascular. This condition proved to be associated with the connective tissue hyperplasia in which, as described in the earlier paper, part or the whole of the musculature of the limb was locally replaced by a loose connective tissue in which blood vessels were scarce, and in which many of those which were present were structurally abnormal and contained few or no corpuscles. There were seven limbs having mid-diaphyseal thickenings which were partly or wholly vascularized, indeed in some hyperaemic, and in each case the connective tissue of the limb was either not hyperplastic or only slightly so. It thus seemed likely that the avascular condition of the new bone was a result of the similar condition in the soft parts of the limb, from which the normal periosteum probably receives at least many of its blood vessels. It may be noted that the vascularity of the old diaphyseal wall and of the marrow was not affected in this condition.

The seven limbs just mentioned, which had well vascularized subperiosteal thickenings, were from animals that lived on the experimental diets for the following periods: 22, 23, 37, 39, 55 (two animals) and 77 days. This seemed to suggest that thickenings when first formed might be well vascularized and lose their vessels later, perhaps with the development of the connective tissue hyperplasia. We therefore studied the vascularization of the developing thickenings in the tibiae of dietetically normal animals having fractured fibulae (material from Exp. 1, see Table 1, Part 1), and of the partially deficient animals in Exp. 9 (see Table 1, Part 1). In the dietetically normal animals killed at 4 and 5 days after the operation, the tissue of the widened cambial layer of the periosteum was almost avascular, the vessels of the periosteum having failed to keep pace with its growth. At 6 and 7 days, the tissue, in which young bone was developing, showed sparse capillary vessels containing blood corpuscles, and by 8 days the new tissue was well vascularized. Thus the early failure of the periosteal vascular system to keep pace with the changes in the cambial zone was quickly rectified. In later stages, the thickenings of the dietetically normal animals were always well vascularized by blood vessels between the trabeculae.

or, in the oldest specimens, in vascular canals in the now compact bone. In the partially deficient animals of Exp 9, on the other hand, the vessels of the periosteum failed altogether to grow *pari passu* with the thickening of the cambial layer and the growth of the new bone, which therefore developed from the beginning in an almost completely avascular bed. It should be added that, in all cases in Exp 9 in which new bone formed, the soft tissues of the limb either showed hyperplasia of the connective tissue, or degenerative changes in the muscles which, as described in the earlier paper of this series, are known to precede the hyperplasia.

The formation of new bone in an avascular bed has theoretical interest, for the observation that, in normal callus formation, cartilage appears in avascular regions while bone forms in regions well supplied with vessels, has led authors (e.g. Wunmbach, 1928) to regard vascularization and the lack of it as the factor determining the differentiation of the two sorts of tissue. The present observation shows that a rich vascular supply is not a necessary condition of bone formation.

Fine structure of the new bone

The bone which makes up the trabeculae of the thickenings could usually be seen to be of two kinds which we shall distinguish as 'fibrillar' and 'granular' bone (Pl 3, figs 15-17). Granular bone was found forming a core to the trabeculae, or as isolated islands in them. The surfaces of the trabeculae were covered with fibrillar bone which must, therefore, have been deposited later than the granular. The granular bone had the following character. The lacunae were of large and irregular form, being produced into processes from which it was rarely possible to see that tortuous canalicules ran off into the matrix. Examined with a high power, the matrix revealed itself to contain, or indeed largely to consist of, great numbers of small rounded granules (Pl 3, figs 15, 16). These were basophil, they, or possibly a substance forming a film over their surfaces, stained with haematoxylin, and they thus gave the tissue a tendency to basophilia which helped to accentuate its contrast against the fibrillar bone which covered the granular bone and stained with eosin. The use of Wilder's method for the silver impregnation of collagen fibres showed that the granular bone contained few fibres, and in places apparently none. Examined with the aid of polarized light, the granular bone was not birefringent (after decalcification). The granules were best demonstrated with Giemsa, which gave them a red-purple colour and showed that they lay in a matrix which stained with eosin. Nothing is known of the nature of the granules, they have been observed in various situations by other authors, as by Weidenreich (1930), who considers that each granule represents a site from which the bone mineral, deposited in molar rather than in molecular dispersion, has been dissolved away in decalcification, leaving behind the organic matrix whose staining properties its earlier presence had in some way modified.

The formation of granular bone was not a result of the partial deficiency of vitamin C, for in dietetically normal animals the first bone to appear in callus formation, and in the development of the thickenings, was of this character, and it was then overlaid by fibrillar bone.

The fibrillar bone, in both normal and vitamin C-deficient animals, had the structure and birefringency of normal bone.

Development of mid-diaphyseal thickenings in partially vitamin C-deficient animals

Experience showed that the development of large subperiosteal thickenings was most likely to occur in the tibiae and fibulae of legs whose fibulae had been fractured while the animal was on a diet partially deficient in vitamin C and radiographs and other evidence indicated that early stages would be found in the first 3 weeks after infliction of the injury. Accordingly, Exp 9 was performed. A fibula of each animal was fractured and the animals killed at intervals. The material obtained was studied mostly in transverse sections because we wished to know whether a swelling of the periosteum preceded the appearance of the new bone, and in longitudinal sections it is easy to be deceived into a false impression of the width of the periosteal tissue. We took sections from positions estimated to be close enough to the fracture to be within the region in which we might expect the periosteum to be affected, but not so close to it as to be within the region in which the periosteum was killed. In addition, some series were sectioned longitudinally.

The sections showed that the formation of a thickening can be arbitrarily divided into five stages. Although the histological picture showed that these stages must follow one another, nevertheless, the last stage could be found so soon after the injury that it could not possibly have developed as a result of its infliction, for example, in one of the legs fixed at 3 days after the fracture. In such cases the bony thickening must have begun to develop before the fracture occurred, this is not surprising, for other experiments showed that as great thickenings could develop in partially deficient animals which had not suffered any intended injury as in those which had (see below). A thickening may continue to grow peripherally for many weeks after the operation and in such cases all the stages may be found, from stage 3 just below the periosteum to stage 5 close to the old bone.

The five stages will be described in order.

Stage 1 This stage was seen in material from legs fixed at 3 and 5 days after the fracture. The condition of the periosteum resembled that described in normal animals in the first days after fracture. It appeared detached from the bone and it was often haemorrhagic. This stage was one of passive injury, it began to give way to the events of the second stage in some of the 3-day material. Both tibia and fibula often showed signs of resorption at their surfaces, with osteoclasts in Howship's lacunae.

Stage 2 This stage was characterized by the formation of a loose and oedematous connective tissue beneath the fibrous layer of the periosteum and filling the space between it and the surface of the bone (Pl 3, fig 18). The tissue consisted of rather star-like cells in a delicate fibrillar stroma, it might show a radial orientation of its fibrils and cells, recalling that described above for the bony trabeculae soon to be formed, or it might show little or no regularity. Many of the cells were in mitosis. Cell divisions occurred much less frequently, if at all, in the fibrous layer of the periosteum, but the histological picture suggested that the fibrous layer might loosen on its inner face and some of its cells be added to the tissue below. Because of its position between the bone surface and the fibrous layer of the periosteum, this tissue was evidently an enlarged periosteal cambium.

Evidently, the enlarged cambium had formed in the space created in stage 1 by

the separation of the periosteum from the bone. Such a space could only exist if either the periosteum had stretched to cover a longer circumference or if the circumference of the bone had lessened, or if both occurred. It is certain that there was in many cases a decrease in the circumference of the bone, for the histological picture sometimes showed very clear evidence that there had been resorption of bone from the surface, the surface was jagged and osteoclasts were seen lying in Howship's lacunae (Pl 3, fig 19). The occurrence of such superficial resorption was, however, not invariable, and even if it might sometimes be sufficient to account for a rather narrow although widened zone, formed within a few days of the injury, between the fibrous layer and the old bone, it certainly could not account for the great width of the space which the new bone eventually occupied. In order to do so, the resorptive process would have had to remove the whole thickness of the original diaphyseal wall, which did not happen. There must, therefore, have been both a reduction in the width of the bone, caused by superficial resorption, and an increase in the circumference of the periosteum, and it is the latter which accounts for by far the larger part of the space eventually occupied by new bone.

Stage 3 In the diffuse mesenchyme-like connective tissue into which the cambial zone of the periosteum transformed itself in stage 2 there now appeared groups of larger cells with dense cytoplasm and rather big vesicular nuclei (Pl 4, fig 20). These were found near the fibrous layer, near the bone, or anywhere between the two. They were osteoblasts concerned in the formation of the new bone, but for their position and known later history they would not have been recognized as osteoblasts, being still more like fibroblasts than like the osteoblasts of later stages in bone development. They seemed to be formed by a differentiation from other cells resembling mesenchyme cells or fibroblasts, rather than by the division of pre-existing differentiated osteoblasts, for there were all transitions between them and the other cells of the widened cambial zone, and between them and the flattened cells of the fibrous layer.

Stage 4 In this stage there began the formation of a tissue which was evidently identical with the granular bone which we have described as forming the cores of trabeculae in later stages, and which was also the first bone formed in the callus of fracture repair in normal animals. It appeared among and between the cells described above as forming groups in the widened cambial zone, and seemed to consist of a homogeneous or fibrillar matrix in which the basophil granules were deposited. It was usual for the group of cells among which the granular bone formed to be arranged radially (Pl 4, fig 21), thus forecasting the radiating architecture seen in the new bone in later stages.

Stage 5 During osteogenesis in the enlarged cambial zone, the formation of granules was a feature of early stages, and at any one point on a developing trabecula soon gave place to the deposition of fibrillar non-granular bone such as is found in the normal skeleton. This was the beginning of stage 5. Very often granular and non-granular bone were separated by a cement layer, but this was not always so, and sometimes a few granules could be found in the young non-granular bone, one might thus be led to suppose granular bone to be a stage in the formation of fibrillar bone, or vice versa. This was refuted by the persistence, in animals killed 3 months after fracture, of granular bone in the cores of the trabeculae, while the more recently

formed bone covering their surfaces was non-granular. The opposite suggestion, that non-granular bone developed into granular, or that the granules formed in it when it became necrotic, is put out of court by the early formation of granular bone, with the granules present from the first, before the fibrillar bone appeared.

The cells concerned in the formation of non-granular bone were often regular series of typical osteoblasts, and were always more osteoblast-like than those found on the surface of developing granular bone.

Porosis and the new bone

The well-known porosis of scurvy (Pl 2, fig 9) had appeared in all the sectioned bones of partially deficient animals of Exps 3-6, except in two or three from animals which died before it developed. It was usually considerable, the old diaphyseal wall showing many large and irregular cavities. Sometimes the porosis was so great as almost totally to destroy the old diaphyseal wall. Osteoclasts were often present, attached to the walls of the cavities, and suggesting that resorption was still going on, but in other cases the cavities showed that there had been deposition of bone on their walls and rows of osteoblast-like cells supported this. One would be inclined to suppose that the resorption must have occurred during the days of total deprivation of the vitamin, and that the deposition had been made possible later, when limited supplies of ascorbic acid were allowed, but this is refuted by the absence of porosis in those which died soonest, and by Exp 9 (see Table 1, Part 1), in which porosis did not begin till long after the end of the total deprivation.

One of the most remarkable features of the histological picture was the limitation of porosis to the old bone, the new bone being free of it and showing few or no osteoclasts. This does not mean that resorptive processes were completely absent from the new bone, for occasionally one could find indications that slight resorption had occurred, but that the large cavities made in the old bone were not seen in the new, and that what resorption did occur was balanced by deposition. Exp 9, in which Exps 5 and 6 were repeated with only unimportant differences, except that the animals were killed sooner and at regular intervals, and that groups 5 and 6 were omitted, threw light on the time relations between the formation of the thickenings and the development of the porosis. In this experiment, in which the last animal was killed at 44 days, porosis never amounted, in the tibia, to more than the appearance of a few large holes seen in cross-sections of the wall, we did not study the fibula in this respect, thinking that the presence of a fracture in that bone would probably influence the onset of porosis in it. Our sections revealed no evidence that porosis had begun in any of the three animals killed at or before 19 days but suggested that it was beginning in two of those killed at 31 days, and in nine out of the ten killed at 34 days and later. Subperiosteal thickenings were found in all animals killed at 37 days and later, and in some of those killed earlier. Thus, the development of the thickenings occurred simultaneously with early stages in the porosis of the old bone. It is, however, true that the development of the new bone was well advanced by the end of the experiment at 44 days, and at this time the porosis was still only beginning, and most of the new bone seems to have been formed before the active porosis whose results were seen in the animals kept for much longer periods (Exps 3-6). In most of this older material, the histological picture

suggested that, at the time of fixation, the trabeculae were no longer growing in length though there might be evidence of slow deposition of fibrillar bone on the surfaces of the trabeculae. Nevertheless, in some examples, the sections showed quite clearly that the subperiosteal trabeculae were still growing at death, although the old diaphyseal wall was riddled with large cavities and some of these were being enlarged by osteoclasts.

Thus the new bone is not only immune, at least relatively, from the porosis which destroys the old, but can continue to be formed even when the porosis of the old is advanced. This may be connected with the poor vascular supply of the new bone, but we could not find satisfactory evidence on this point.

Diaphyseal thickening and injury to the bone

The difference between the thickenings formed on the tibia and fibula of dietetically normal animals with fractured fibulae, and the much greater development of new bone, worthy to be called 'hyperostosis', which appeared in many animals on diets which were partially deficient in vitamin C, was quantitative and not qualitative. The difference between the two dietary groups was in the thickness of the new bone which in the animals on partially deficient diets usually greatly exceeded that in the dietetically normal animals. In addition, the trabeculae of the new bone were usually thinner, longer, more regularly orientated, and more sharply differentiated into granular and fibrillar bone in animals on partially deficient diets than in those on normal diets.

Thus the partial deficiency of vitamin C tended to cause an exaggeration of a structural change which, in dietetically normal animals, did not occur at all except after operative interference. The combined action of the two factors is emphasized by comparing Exps 3 and 4, in which the deficiency did not begin till 15 days after the infliction of the injury, with Exps 5 and 6, in which the deficiency began more than 3 weeks before the injury. When the time of origin and the growth of the thickenings were studied in the radiographs it was found that in Exps 3 and 4 the thickenings began soon after the infliction of the injury, and before the diet was made partially deficient (as in animals on fully supplemented diets), but only went on to become extreme after the supply of vitamin C was reduced. That is to say, the relatively slight thickenings which formed after the operation, on the fully supplemented diet, went on to become hyperostoses under the influence of a partial deficiency of vitamin C. In Exps 5 and 6, on the other hand, the partial deficiency of the vitamin did not induce the formation of thickenings until after the operation, but the thickenings which then formed tended to become extreme more rapidly than did those in Exps 3 and 4.

It was thus the operation which determined the occurrence of the thickening at all, but the partial deficiency of vitamin C which caused its enlargement into a 'hyperostosis'.

In both dietetically normal and partially deficient animals it appeared to be unimportant in the production of the thickenings whether the attempt to fracture the fibula was successful or not. Combining all dietetic groups of Exps 3-6, the fibula was successfully fractured in thirty-two limbs and, among these, subperiosteal thickenings formed in eighteen, while the attempt to fracture the fibula failed in

thirty limbs and subperiosteal thickenings formed in fourteen. The difference is obviously insignificant.

Although in animals on normal diets there was never any development of subperiosteal thickenings on the bones of unoperated legs, this did occur among the partially deficient animals. In Exps 3 and 4, out of twenty-five operated legs of partially deficient animals, ten (40%) showed marked mid-diaphyseal thickenings of the tibia, fibula, or both, and out of eleven unoperated legs such thickenings occurred in two (18%). In Exps 5 and 6, out of nineteen operated legs, thirteen (68%) showed mid-diaphyseal thickenings, and six (17%) unoperated legs out of thirty-five showed similar thickenings. In Mouriquand's experiments there was no operative interference.

Formation of the widened cambium

We have seen that the formation of the widened periosteal cambium is a result of the detachment of the periosteum from the bone. There was an obvious resemblance between mid-diaphyseal thickenings and the callus which forms around a fracture in an otherwise normal animal, indeed, when such a callus was present the thickening was an extension of it. A number of students of fracture repair have emphasized the importance of periosteal detachment for callus formation. Thus, Lever (1922), Koch (1924) and Haldeman (1932) regard its separation from the cortical bone as a necessary condition of callus formation, and the first two, if not the third, consider the amount of callus formed to be proportional to the degree of the separation, always provided that the periosteum retains its connexion with the surrounding soft tissues from which it receives most of its blood supply. It is well known that periosteal detachment occurs in acute scurvy. Meyer (1928), in his study of acute scurvy in guinea-pigs, commented that 'the periosteum usually can be stripped with greatest ease', mentioning particularly the mandible. We examined bones of three animals in acute scurvy, decalcifying three tibiae and examining cross-sections cut with a razor, the periosteum appeared to be completely detached over wide areas. No injury had been inflicted. Meyer attributed the separation of the mandible from the periosteum to resorption of bone from the surface, and, as stated elsewhere, we found that such resorption occurred from the surfaces of limb bones. It is perhaps significant that in Exp 9 (see Table 1, Part 1) the transverse sections of tibiae of animals killed in the first 5 days after fracture of the fibula showed the periosteum detached from the tibia, this had occurred before embedding and sectioning, for it was seen in the decalcified material before these processes were begun, and was therefore not an artefact caused by them. Later, after the development of the enlarged cambial tissue, no detachment was seen. One cannot help suspecting that it may have been caused by resorption of superficial layers of the bone, after operation, re-attachment occurred when the subperiosteal space was filled by the enlarged cambium.

Because of the known inability of scorbutic animals to make collagen fibres, by which the fibrous layer of the periosteum is attached to the bone, it is easy to understand how, even in a moderate scurvy, this layer might have become detached from the bone by any factor tending to have such an effect, and would then have been less readily attached to it again. Among such factors may perhaps be included the fracture forceps, whose use may perhaps drag on the periosteum of both bones.

tending to detach it. However, the fracture forceps can have been only a contributory factor, for the thickenings sometimes developed when there had been no operation. This only happened in partially deficient animals, in both our experiments and those of Mouriquand. We think it possible that a mechanical factor operating in these (and other) cases may have been the pull of muscles on the periosteum, especially when the animals resisted anaesthesia, before the taking of the radiographs, by vigorous kicking. This is perhaps supported by the frequent observation of thickenings which had grown in steps, and showed an older zone of trabeculae which had been closed peripherally by a shell, and external to this a new series of younger trabeculae radiating towards the fibrous layer. Each such 'step' could have been caused at the taking of one of the photographs. Folis (1948) has shown that the immobilization of a limb prevents the microfractures which, in scorbutic animals, otherwise occur close to the epiphyses.

In addition to the direct action of the forceps and contracting muscles, and perhaps more immediately important than either, was the oedema which followed injury to the fibula and which provided a fluid which, along with some haemorrhage, occupied the widened subperiosteal space.

We know that the mid-diaphyseal thickenings occurring in the tibiae of dietetically normal animals after operation were produced by the same detachment, oedema, cambial proliferation and bone formation, as in the partially deficient animals, and we must suppose that its lesser degree reflected a periosteum whose collagen fibres were less readily broken and more readily reformed, a bone whose surface layers were less readily resorbed, and a vascular and metabolic condition in which the oedema caused by injury was less, and was more rapidly drained away, than in animals provided with ample vitamin C.

The determination of the architecture of the new bone

The formation of an abnormal bony architecture of a regular character makes it possible to investigate the conditions of its formation in the hope of throwing light on the mechanism which brings about the development of such structures. Such a regular architecture is provided by the radiating trabeculae of the mid-diaphyseal thickenings.

We have here two problems: (a) that of the factors determining whether periosteal growth of a bone in thickness shall be by the apposition of new compact bone, as normally happens, or by the formation of trabecular bone, as in our thickenings, and (b) that of the factors determining the orientation of the trabeculae.

In normal growth in width of the diaphysis, new compact bone is deposited by a periosteal cambium which consists of little, if anything, more than a single layer of osteoblasts, with the fibrous layer immediately outside it. Similarly, when in one of the subperiosteal thickenings the growth of the trabeculae has brought their ends against the fibrous layer of the periosteum, the circumferential bony shell is formed (Pl 3, fig 14), and, evidently, continued growth of the shell would produce a zone of compact bone. Indeed, after return to a diet containing ample supplies of ascorbic acid, circumferential layers of compact bone are formed (see below). In the formation of both the 'shell' and of these layers of compact bone, the condition of the periosteum resembles the normal: the bone is formed by a narrow cambial layer

beneath an immediately apposed fibrous layer. Thus, the formation of trabecular or of compact bone depends on the dimensions of the tissue in which the bone is developing. If osteogenetic cells are scattered, as it were in three dimensions, through a widened cambial tissue, trabecular bone is formed, but if the fibrous layer of the periosteum holds them in a single sheet against existing bone, they must evidently give rise to compact bone.

A second factor affecting the compact or trabecular nature of the bone in these experiments is the nutritional state. Thus, the trabecular new bone of the thickenings was made compact when the supplies of ascorbic acid were increased to 10 mg daily, and the compact bone of the diaphysis was made trabecular by porosis during the prolonged partial deficiency.

The walls of long bones are compact because the periosteal deposition of bone here occurs in a narrow cambium held against the old bone, while endosteal bone, unless its formation is limited to apposition against the inner surface of the diaphyseal wall, is usually trabecular.

Turning to the problem of the orientation of the radiating trabeculae of the thickenings, one of us (Murray & Selby, 1930, Murray, 1936) has earlier discussed the formation of similar radiating architectures in other bones (the limb bones of normal chick embryos, of chick embryo limb bones becoming bent while developing in chorio-allantoic grafts, and the bent limbs of chondrodystrophic chickens, Landauer, 1927). The opinion was expressed that the radial orientation of the trabeculae was in those instances to be referred, as Studitski (1934) suggested in some of his experiments with chorio-allantoic grafts, to tensions set up, by the growth in width of the epiphyses and the curvature of the shafts, between the fibrous layer of the periosteum and the cartilaginous or bony shaft. When that discussion was written, however, there was very little evidence that such tensions existed at all. Sections made from the material of Exp 9 (see Table 1, Part 1), in which we studied the development of the new bone in animals on partially vitamin-C deficient diets, showed young trabeculae of bone radiating from the old diaphyseal wall towards the fibrous layer of the periosteum, but ending at various points in the widened cambium, and then being continued to the fibrous layer by strands of collagen fibres (Pl 4, fig 22, arrows) about which were grouped cells which were shown, by comparison with those on the bony parts of the trabeculae, to be osteoblasts. The histological picture left no room for doubt that the bony trabeculae were growing outwards along the collagen fibres and owed their radial arrangement to that of the collagen fibres which provided them with a scaffolding. It has been shown by a number of workers (e.g. Weiss, 1928, 1929, 1933, Levy, 1904) that collagen fibres develop along lines of tension. In our material, the oedematous condition of the subperiosteal tissue separated the periosteum from the bone and set up between the two just such tensions as Studitski imagines, and along these tensions the collagen fibres developed. Finally, the sections show that it is along the radiating collagen fibres that the bony trabeculae form.

The effect of restoring adequate supplies of vitamin C

In Exp 7 (see Table 1 of Part 1) the guinea-pigs, after deprivation of ascorbic acid for 10 days, were allowed 0.5 mg ascorbic acid daily for 55 days, and thereafter were given 10 mg of ascorbic acid, and cabbage, daily until they were killed. We then

investigated the thickenings which had been first formed in the period of partial deficiency. Since we had not at the time appreciated the importance of injury in causing subperiosteal thickening, no fractures were inflicted, this was unfortunate because it reduced the number of markedly thickened bones available for study after 'cure' of the partial deficiency. We obtained sections of four tibio-fibulae from three animals. These all showed thickenings with the usual radiating architecture, but they differed from those seen in animals which died or were killed during the partial deficiency in the much greater thickness of the trabeculae, and corresponding reduction of the intertrabecular tissue. The originally trabecular thickenings had in fact been transformed into compact bone, while retaining clear indication of their original architectural character (Pl 4, fig 23). This change was brought about by the deposition of additional fibrillar bone on the surfaces of the trabeculae. Thus, the chief effect of the additional vitamin C on the new bone was to increase its density.

There was, however, another effect which was not seen in animals which were killed, or died, while still on the partially deficient diet. This was the appearance, in the consolidated mass of new bone, of a small number of holes (Pl 4, fig 24) obviously made by resorption and often containing osteoclasts. These holes were present in all four specimens, and in size and number recalled the early stages in scorbutic porosis seen in animals of Exp 9 (see Table 1, Part 1). The amount of bone which had been removed must have been negligible, but of course it might have become a more important quantity if the animals had been allowed to live longer.

When the superficial region of the bone, close beneath the periosteum, was examined, we found clear evidence that its thickness had been reduced by resorption from the surface (Pl 4, fig 25). This was very obvious in one specimen particularly, in which the radiating trabeculae were sharply cut off just beneath the periosteum, where osteoclasts were present, and the picture was one typical of a resorption surface.

In parts of this specimen, and in large areas of the surfaces of others, the abrupt and jagged peripheral ends of the partly resorbed trabeculae did not reach the surface, but were buried beneath a superficial layer of bone developed as a circumferential subperiosteal sheet (Pl 4, fig 23), such as is deposited on the surfaces of bones in normal growth. This superficial bone must have been formed after the development of the trabecular thickenings, because it overlay a surface from which part of the trabecular bone had obviously been resorbed (like an 'unconformity' in Geology). Since nothing of the sort was ever seen in animals killed while on the partially deficient diet, it must have been formed after the enrichment of the diet with the full supplement of the vitamin.

Turning to the bone lining the marrow cavity, we expected to find the original diaphyseal cylinder but with the porosis cavities more or less completely filled with new bone. Instead, we found compact bone showing no convincing sign that it had ever suffered porosis at all, and this bone covered sharply broken off and jagged inner ends of the trabeculae (Pl 4, fig 23). It is evident that, at some time after the formation of the trabeculae, resorption from the marrow cavity destroyed both the old bone of the diaphyseal wall (which must have been porotic) and the inner ends of the trabeculae. This may have happened during the partial deficiency, for we have

other specimens fixed during this period in which endosteal resorption had removed almost the whole of the original cylinder, leaving a wall composed entirely of radiating trabeculae (Pl 3, fig 13). Of course, resorption from the marrow is a necessary concomitant of normal growth. After this resorption, the new internal compact bone was then formed.

Thus, the restoration of normal dietary conditions led to a strengthening of the bone composing the shaft by the conversion of trabecular into compact bone, and to a reduction of the abnormal thickening of the wall by resorption from both its outer and inner surfaces. That such a reduction in thickness of the wall did actually occur was confirmed by the radiographs. It was not reversed by some increase in thickness caused by the formation of compact bone both internally and externally, and which may reflect a complex remodelling of the form of the bone as a whole. It must be remembered that, so long as an element is growing in length, the bone at any level in the shaft is in process of becoming the bone at another level (i.e. further from the growth cartilage which formed it), and that the width of the shaft, the thickness of the bony wall, and the width of the marrow cavity, are different at different levels. In particular, the wall becomes thicker and the marrow cavity narrower, as the distance from the growth cartilage increases, facts which may be connected with the formation of the new compact bone, on inner and outer surfaces, described above.

SUMMARY

1 After fracture, or attempted fracture, of the fibula, guinea-pigs on normal diets showed formation of new bone on the surfaces of the diaphyses of both tibia and fibula of the operated leg.

2 Guinea-pigs which were made partially deficient in vitamin C either before, or after, the operation, showed similar but often enormously larger thickenings.

3 In dietetically normal animals the thickenings never developed except after operation, and in partially deficient animals the thickenings occurred more frequently when the operation had been performed, but also occurred when there had been no operation.

4 The porosis of scurvy was seen in the old bone of the original diaphyseal wall but not in the new bone of the thickenings.

5 In both dietetically normal, and partially deficient animals, the new bone was trabecular in structure, not compact like the old diaphyseal bone. The trabeculae radiated from the surface of the bone outwards to the fibrous layer of the periosteum. Since their extent was much greater longitudinally than circumferentially, they were really narrow and irregular longitudinal ridges.

6 The thickenings developed in a widened periosteal cambium which formed when the fibrous layer of the periosteum had been separated from the bone beneath it by the post-operative oedema, by direct mechanical action in the operation itself, or by muscular action. In the partially deficient animals, the greater thickness of the new bone reflected the easier detachment of the periosteum and its less ready reattachment, a greater tendency to superficial resorption of old bone, and the greater and more lasting oedema caused by the injury, than occurred in animals on normal diets.

7 The thickenings were sometimes well vascularized by vessels between the trabeculae, but more often were almost avascular. This condition of the new bone was associated with, and probably caused by, the avascular state of the hyperplastic connective tissue which developed in place of the degenerate musculature (see the first paper of this series). The thickenings which developed in dietetically normal animals after operation were quickly supplied with vessels, but in the partially vitamin C-deficient animals the new bone, like the new connective tissue, usually did not become vascularized, or only very poorly so. A richly vascular bed is not a necessary condition of bone formation.

8 The factors determining the formation of compact or trabecular bone, and of the radiating architecture of the new bone, are discussed. When osteoblasts were scattered through a wide cambial tissue, trabecular bone was formed, but when a thin layer of osteoblasts was held against the old bone by a closely attached fibrous layer, the new bone was compact. The radiating architecture of the new bone was determined by a pre-existing radial arrangement of the collagen fibres in the widened cambium. It is argued that the orientation of the collagen fibres was determined by tensions, set up by the oedema, between the surface of the old bone and the fibrous layer of the periosteum.

9 When animals which had been kept for long periods on diets partially deficient in vitamin C, and which had developed trabecular thickenings, were given ample supplies of the vitamin, the addition of new bone to the surfaces of the trabeculae converted the trabecular bone into compact bone, while resorption from both periosteal and endosteal surfaces of the bone reduced its thickness. The reduction in thickness by superficial resorption was complicated by the deposition of new compact circumferential bone, whose formation may have been connected with form changes normally associated with growth.

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ABBREVIATIONS

<i>camb</i>	cambial zone of periosteum	<i>haem</i>	haemorrhage
<i>circ lam</i>	circumferential lamellae	<i>mar</i>	marrow
<i>con th</i>	consolidated thickening	<i>n b</i>	new bone
<i>fib</i>	fibula	<i>o b</i>	old bone
<i>fib b</i>	fibrillar bone	<i>peri</i>	periosteum
<i>fib per</i>	fibrous layer of periosteum	<i>por</i>	porosis cavity
<i>gran b</i>	granular bone	<i>shell</i>	circumferential bony shell

PLATE 1

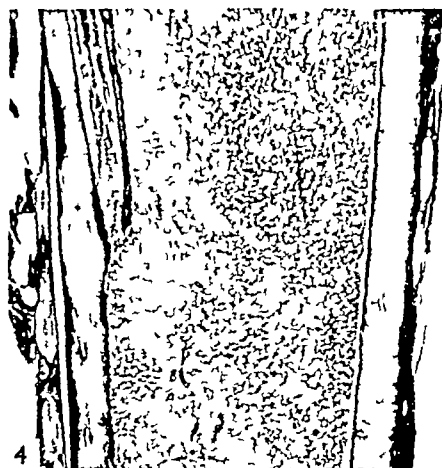
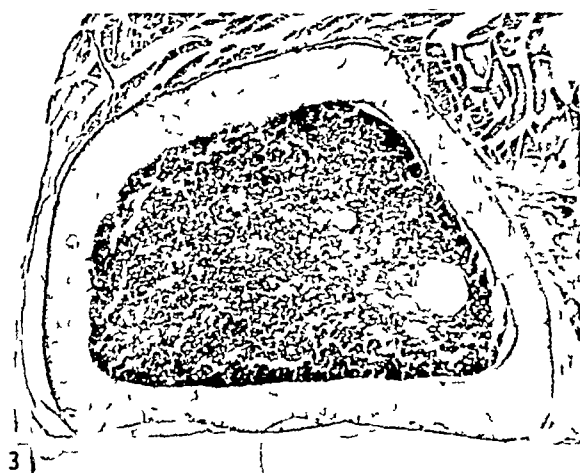
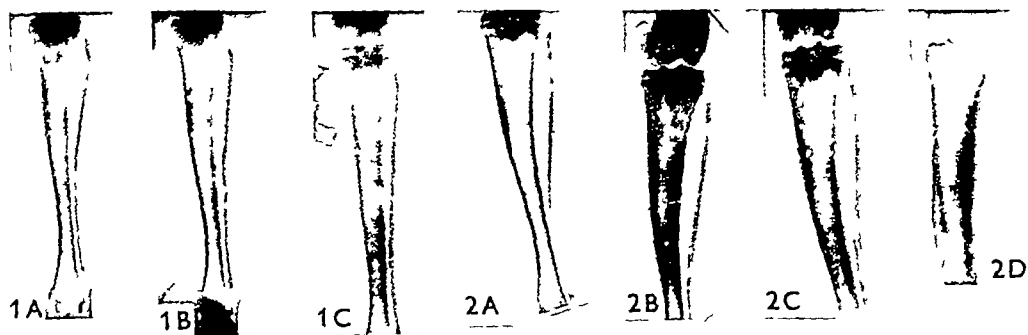
- Fig 1 Normal guinea pig Development of subperiosteal thickening on lateral wall of tibia after fracture of fibula A, day of fracture, B, 19th day, C, 50th day after fracture
- Fig 2 Guinea pig at first on normal diet, later on partially vitamin C deficient diet Left tibia and fibula From Exp 4, group 4 A, day of operation, normal diet, B, 15th day after operation, 1st day of partially deficient diet, C, 28th day after operation, 14th day of partially deficient diet, D, 81st day after operation, 67th day of partially deficient diet Tibial and fibular thickenings, at first slight, become greatly enlarged after prolonged partially deficient diet
- Fig 3 Normal guinea pig Transverse section of normal tibia Haematoxylin and eosin $\times 15$
- Fig 4 Normal guinea pig Part of longitudinal section of normal tibia Azan $\times 15$
- Fig 5 Normal guinea pig Part of longitudinal section of diaphyseal wall of tibia, showing new trabecular subperiosteal bone, 16th day after fracture of fibula Haematoxylin and eosin $\times 41$
- Fig 6 Normal guinea pig Part of longitudinal section of diaphyseal wall of tibia, 3rd day after fracture of fibula Fibrous layer of periosteum lifted from bone by haemorrhage Haematoxylin and eosin $\times 195$
- Fig 7 Normal guinea pig Part of longitudinal section of diaphyseal wall of tibia, 4th day after fracture of fibula Space beneath fibrous layer of periosteum filled by cells Azan $\times 195$

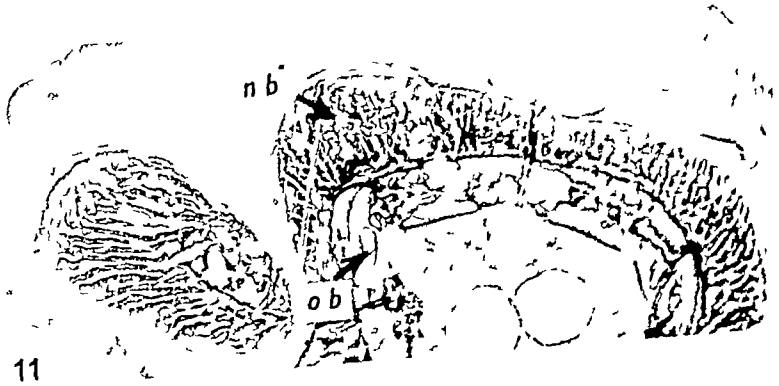
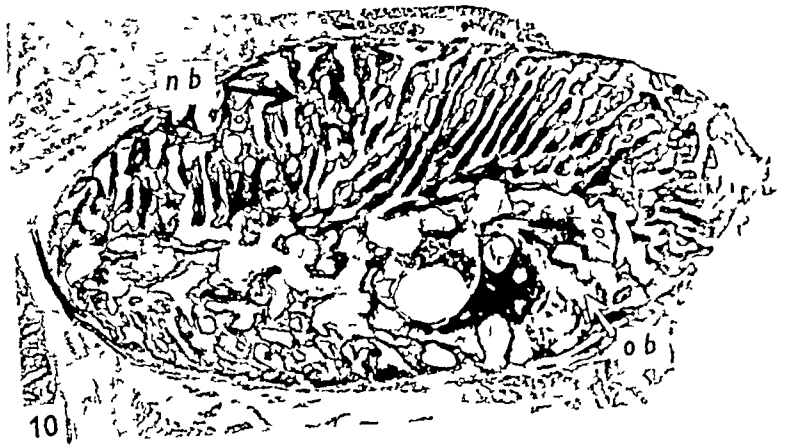
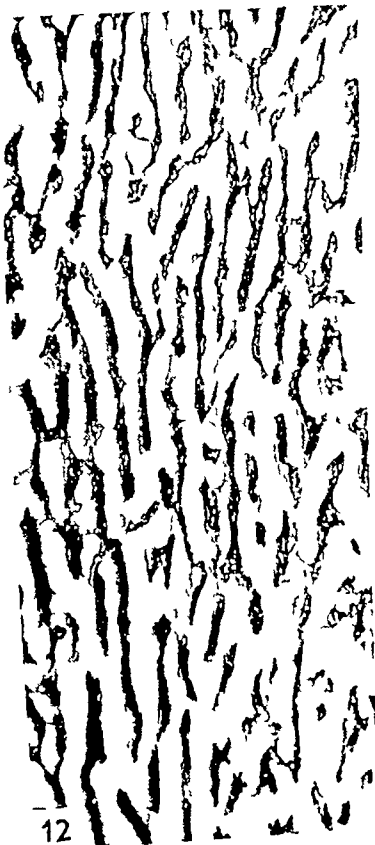
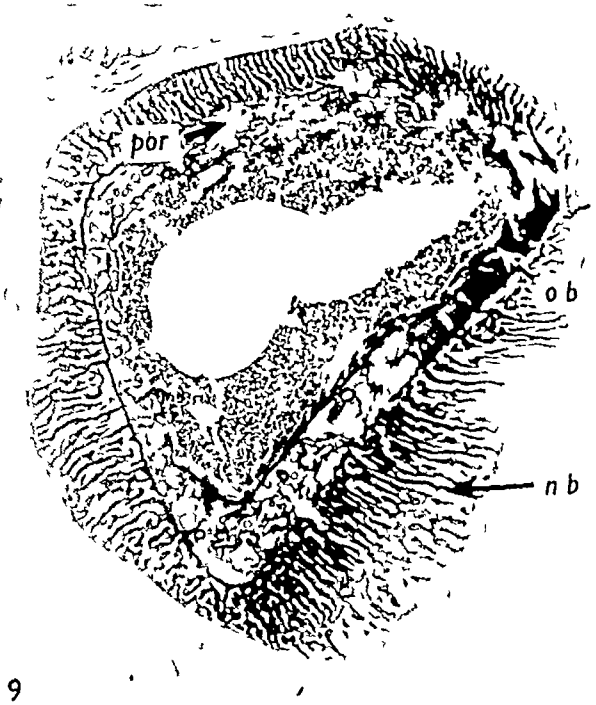
PLATE 2

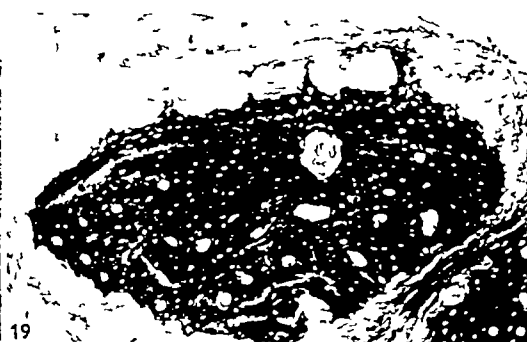
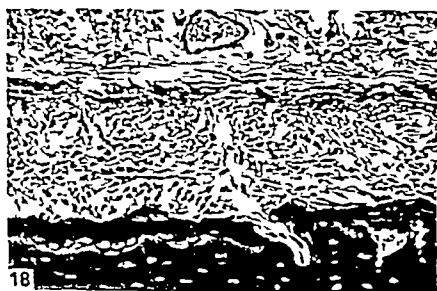
- Fig 8 Normal guinea pig Part of longitudinal section of diaphyseal wall of tibia, 5th day after fracture of fibula Formation of new trabecular bone in the widened cambium Haematoxylin and eosin $\times 195$
- Fig 9 Partially vitamin C deficient guinea pig Transverse section of tibia showing great subperiosteal thickening, and porosis of the old bone Haematoxylin and eosin $\times 95$ From Exp 4, group 4, 81st day of experiment, 67th day of partially deficient diet
- Fig 10 Partially vitamin C deficient guinea pig Transverse section of fibula showing subperiosteal thickening Haematoxylin and eosin $\times 31$ From Exp 3, group 3, 101st day of experiment, 86th day of partially deficient diet
- Fig 11 Partially vitamin C deficient guinea pig Transverse section of tibia and fibula, to show the two types of architecture (compact old bone, partly eroded by porosis, and trabecular new bone) separated by a darkly staining cement line Haematoxylin and eosin $\times 17$ From Exp 3, group 3, same animal as Fig 10
- Fig 12 Partially vitamin C deficient guinea pig Part of a tangential section of the tibial wall, passing through the new trabeculae, showing that what appear in transverse sections to be radial spokes are really narrow ridges Haematoxylin and eosin $\times 48$ From the same specimen as Fig 9

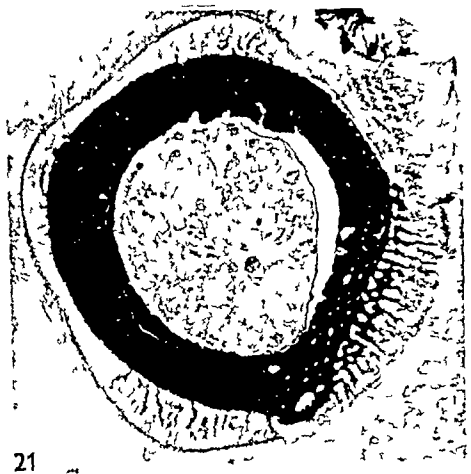
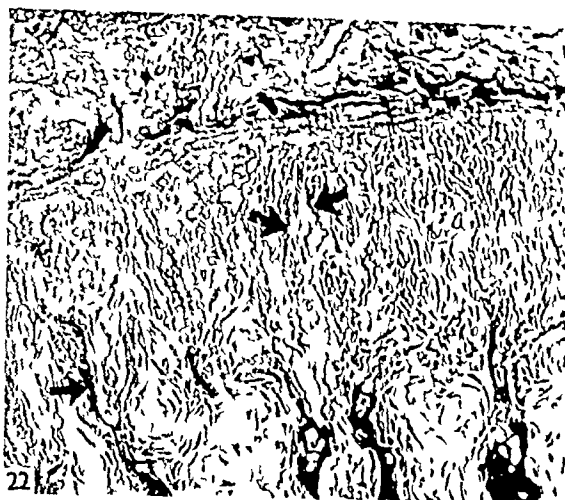
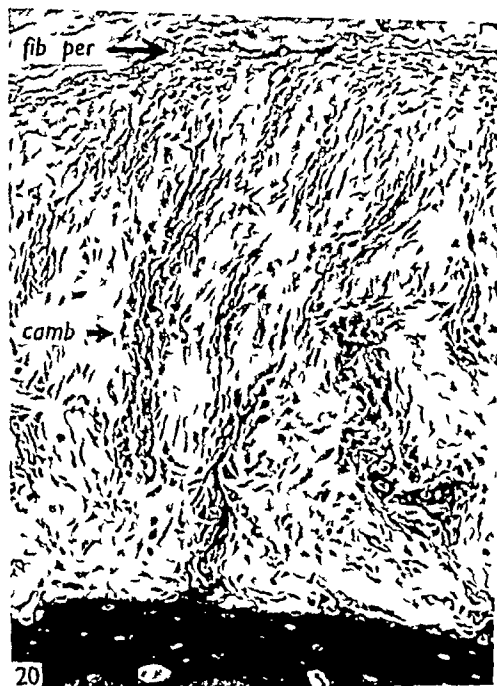
PLATE 3

- Fig 13 Partially vitamin C deficient guinea pig Part of a longitudinal section of the tibia, showing the diaphyseal wall composed entirely of new trabecular bone with a paper thin zone of old compact bone remaining (bordering the marrow cavity, below) Haematoxylin and eosin $\times 65$ From Exp 6, group 4, 85th day after beginning the experimental diet, and 62nd day after the operation
- Fig 14 Partially vitamin C deficient guinea pig Part of a transverse section of the tibia, showing the radiating trabeculae of the thickening joined at the surface by a thin shell of circumferential bone Haematoxylin and eosin $\times 74$ From Exp 3, group 3, the same animal as Figs 10 and 11
- Fig 15 Partially vitamin C deficient guinea pig An area of the subperiosteal thickening showing granular bone, notice the large lacunae and granular matrix Haematoxylin and eosin $\times 910$ From Exp 3, group 3, 101st day after operation, 86th day after beginning the experimental diets









- Fig 16 Partially vitamin C deficient guinea-pig Part of a tangential section of the tibial wall, passing through new trabeculae, showing granular (dark) and fibrillar (pale) bone Haematoxylin and eosin $\times 185$ From the same specimen as Fig 9
- Fig 17 Partially vitamin C deficient guinea pig Part of a trabecula of new bone in a subperiosteal thickening, impregnated with silver by Wilder's method and stained with haematoxylin The granular bone at the core of the trabecula is almost without fibres while the fibrillar bone covering its surface is densely fibrous $\times 940$ From Exp 3, group 3, the same animal as Fig 10
- Fig 18 Partially vitamin C deficient guinea pig Part of a transverse section of the tibia, showing the fibrous layer lifted from the bone and the space beneath filled by an enlarged cambial tissue Azan. $\times 195$ From Exp 9, 6th day after operation, 31st day after beginning the experimental diet
- Fig 19 Partially vitamin C deficient guinea pig Transverse section of fibula, showing evidence of superficial resorption of the bone, and a widened cambial tissue Azan $\times 90$ From Exp 9 the same animal as Fig 18

PLATE 4

- Fig 20 Partially vitamin C deficient guinea pig Part of a transverse section of the tibia showing an early stage in the development of bone in the widened cambial tissue, with groups of osteoblasts radially arranged Azan $\times 195$ From Exp 9, 12th day after operation and 37th day after beginning experimental diets
- Fig 21 Partially vitamin C deficient guinea pig Transverse section of tibia Widened cambium and developing trabeculae of new bone, radially orientated Azan $\times 20$ From Exp 9 the same animal as Fig 20
- Fig 22 Partially vitamin C deficient guinea pig Part of a transverse section of the tibia, showing the peripheral part of the widened cambial tissue Below, the ends of new bony trabeculae, these are continued to the fibrous layer of the periosteum by strands of collagen fibres (indicated by arrows) $\times 195$ From Exp 9, the same animal as Fig 20
- Fig 23 Part of the tibial wall of a guinea pig which, after prolonged partial vitamin C-deficiency, was cured with ample vitamin C The section shows consolidation of the subperiosteal thickening and new internal and peripheral circumferential lamellae Haematoxylin and eosin $\times 135$ From Exp 7, group 2, 107th day of experiment, 41st day after return to balanced diet, no operation
- Fig 24 Part of the tibial wall of the other leg of the same animal as Fig 23 Consolidation of the thickening, new internal circumferential lamellae and cavities in the thickening formed by resorption Haematoxylin and eosin $\times 92$
- Fig 25 Part of the tibial wall of the same animal as in Figs 23 and 24 Evidence of resorption from the surface of the thickening Haematoxylin and eosin $\times 200$

AN UNUSUAL ANOMALY OF M FLEXOR DIGITORUM LONGUS

BY T E BARLOW

Department of Anatomy, University of Durham, Newcastle upon Tyne

The anomaly occurred in a male subject, aged 71 years, and was bilateral, except for slight differences. The condition on the left side is described first. The tendon of the flexor digitorum longus was normal until it reached the foot. It then divided at the usual point, but into three tendons only. Those to the 2nd and 3rd toes were normal but the tendon to the 4th toe was a small slip forming not more than a quarter of the total number of tendinous fibres reaching that digit, the remainder of the fibres passing to the 4th toe, as well as those to the 5th toe, being derived from an anomalous flexor accessorius.

Flexor digitorum accessorius (quadratus plantae) was composed of three parts, the most superficial of which was a small muscle arising by two heads, a tendinous one from the medial tubercle of the calcaneum just medial to the long plantar ligament and a muscular one from the sheath of flexor hallucis longus and flexor digitorum longus. It joined the lateral side of a long tendon inserted into the terminal phalanx of the little toe and contributed also to the tendon of the 4th toe.

The intermediate part of the muscle was the main source of the tendons to the 4th and 5th toes. It was a triangular sheet arising by fleshy fibres from the under surface of the calcaneum behind the sustentaculum tali as far back as the medial tubercle. Its tendon of insertion split to go to both the 4th and 5th toes, that to the 5th being joined by the fleshy fibres, already mentioned, from the superficial part of the muscle.

The deep part of the muscle arose from the underside of the calcaneum in front of and lateral to the sustentaculum tali and was inserted in the normal manner of the flexor accessorius into the tendon of flexor digitorum longus. The 4th lumbrical arose from the tendons to the 4th and 5th toes.

In the right foot the same condition was found except that the tendon to the 4th toe was derived equally from flexor digitorum longus and accessorius.

The interest of this case lies in the support it gives to the phylogenetic history of the flexor muscles of the toes as interpreted by Wood Jones (1944, pp 219-225).

The underlying factor in this phylogeny is the necessity for the digital flexors to remain contracted, while the extensors also contract, in order to enable the toes of the supporting and propelling foot to push against the ground at the moment of take off in walking. While the flexed toes are pushing against the ground the digital extensors have to act from them to pull the leg forwards over the ankle joint.

The necessary concomitant of extensor contraction is relaxation of the belly of the long flexor, but at the same time the toes must remain flexed against the ground. This conflict of interests has been solved by turning the superficial flexor into an intrinsic muscle of the foot by divorcing it from its belly in the leg (plantaris) and by transferring the work of the deep flexor in this connexion to the flexor accessorius.

The logical conclusion of this story would be to divorce the deep flexor also from its belly in the leg, and the initial stage in such a divorcement appears to be occurring in the present case

The function of flexor accessorius is often said to be to draw the tendons into which it is inserted into a less oblique position

In this connexion it is interesting to note that in the present instance the anomalous tendons to the 4th and 5th toes having separated themselves from the remaining

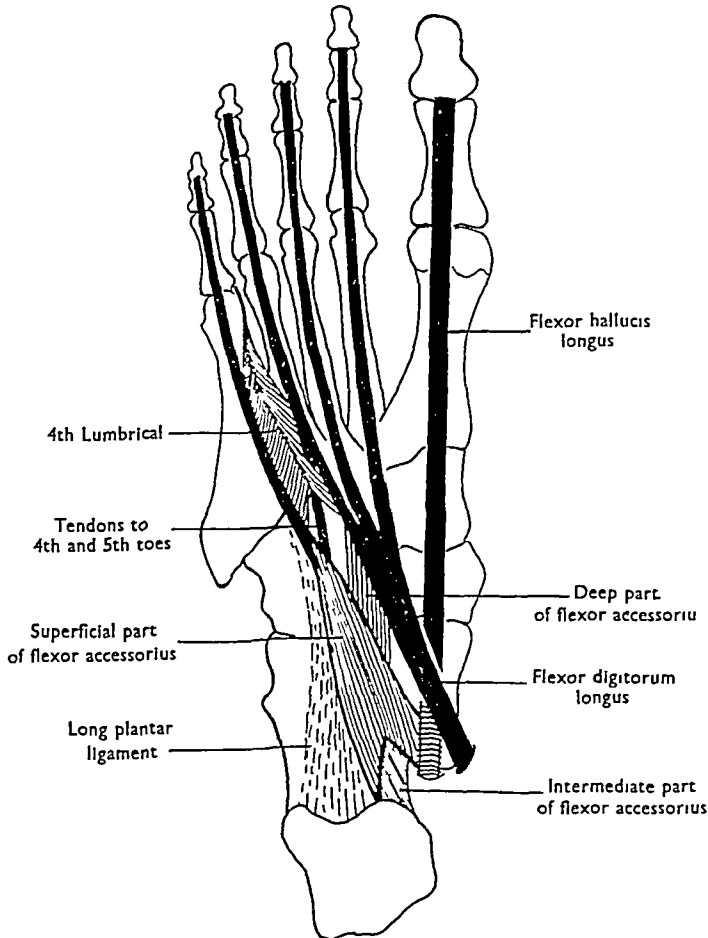


Fig 1

tendons of flexor digitorum longus, not only maintain their original oblique direction but, having obtained a bony attachment to the medial tubercle of the calcaneum, they discard any further assistance from the flexor accessorius, for the lateral head of that muscle is absent

Here, then, is a situation that should help to dispel the idea that living muscular tissue is used instead of the usual fibrous pulley to change the direction of a tendon or group of tendons

SUMMARY

An interesting anomaly of flexor digitorum longus is described and a reason for its presence adduced. A further reason is given for supposing that flexor accessorius (quadratus plantae) does not perform the functions usually ascribed to it.

REFERENCE

JONES, F. WOOD (1944) *Structure and Function as seen in the Foot*. London: Baillière, Tindall and Cox.

ALKALINE PHOSPHATASE ACTIVITY IN NORMAL AND DEGENERATED PERIPHERAL NERVES OF THE RABBIT

By JUNE MARCHANT, *Department of Embryology, University College, London**

INTRODUCTION

The process of nerve degeneration has many features in common with the healing of skin wounds, both involve cell proliferation and migration and the laying down of collagen fibres. Fell & Danielli (1943) using the histochemical technique of Gomori (1939) and Takamatsu (1939), demonstrated alkaline phosphatase activity in healing skin wounds and burns of rats greatly in excess of that found in normal skin. The increased amount of enzyme was situated on invading polymorphs, cell nuclei and newly formed collagen fibres. They suggested that the enzyme associated with the collagen fibres might take part in the 'metabolic processes more intimately concerned in the laying down of collagen', and further support was given to this hypothesis by their more recent work (Danielli, Fell & Kodicek, 1945), which showed that the poor differentiation of collagen fibres in healing skin wounds of scorbutic guinea-pigs was associated with an absence of histochemically demonstrable phosphatase.

Degenerating peripheral nerve provides useful material for testing further this hypothesis of Fell & Danielli. Degenerative processes take place almost uniformly throughout the peripheral stump of such a nerve, which forms a large and clearly defined mass of uniform material for investigation. A considerable amount of collagen is formed during degeneration and the time-course of its formation is better known than in skin, for it has been studied quantitatively by Abercrombie & Johnson (1946*a*). They found, by chemical methods, that the amount of collagen in the degenerating rabbit sciatic nerve increased throughout a period of at least 200 days after the nerve was severed. Furthermore, collagen formation in this tissue is largely dissociated from cell-proliferation, which ceases after 25 days of degeneration (Abercrombie & Johnson, 1946*b*), whereas in skin, so far as present information goes, the two processes largely overlap in time and consequently may confuse interpretation of the associated phosphatase activity. Another convenient feature of degenerating nerve lies in the fact that, except in the very localized region of actual trauma, there is no invasion of the tissue by polymorphs, which might complicate the issue since they are very rich in phosphatase.

If Fell & Danielli are correct in their hypothesis that alkaline phosphatase is concerned in the laying down of new collagen, we may expect to find this enzyme present in degenerating peripheral nerve throughout the prolonged period in which, according to Abercrombie & Johnson, collagen formation occurs.

Studies of alkaline phosphatase in degenerated peripheral nerve do not appear to have been undertaken hitherto. Landow, Kabat & Newman (1942) were unable to demonstrate histochemically the presence of this enzyme in the undegenerated sciatic nerves of cat, mouse and man except in the vascular endothelium, in the chicken the

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Schwann sheath showed a decided reaction, though there was none in axons or the myelin sheath

MATERIAL AND METHOD

The material used for this work was obtained from adult rabbits. Several kinds of undegenerated nerves were studied, namely the sciatic (peroneal and tibial divisions) anterior mesenteric, greater splanchnic and vagus nerves. Degenerated material was provided by the peripheral stump of the severed sciatic nerve. The nerve had previously been cut high up in the thigh under nembutal and ether anaesthesia and the peripheral stump allowed to degenerate for a period of time varying from 2 to 100 days, the rabbit being then killed by air embolism. To prevent re-innervation of the peripheral stump during the longer periods of degeneration (from 50 days onwards) the central stump of the nerve was sutured to the undersurface of the skin. In experiments involving degeneration for 25 days, or less, re-innervation was prevented by removing about 1 cm. of the nerve. Both sciatic nerves of each rabbit were usually severed, but on different occasions, so that at autopsy each animal provided nerves of two different periods of degeneration. In some cases pieces of undegenerated nerve removed at the operation were used for comparison with degenerated nerve from the same rabbit taken at autopsy.

Pieces of nerve 0.5–1.0 cm. long were fixed in 80 or 95% alcohol, fixation in 80% for about 20 hr. giving tissue of the best cutting quality. Some of these pieces were then taken down to water, and frozen sections were cut from them, other pieces were dehydrated, cleared in benzene, embedded in paraffin at 56° C., and longitudinal and transverse sections of each piece were then cut at a thickness of 7 μ . Sections were treated by Danielli's (1946) modification of Gomori's technique, being incubated for 18–22 hr. at 37° C. and pH 9.3 with sodium glycerophosphate in the presence of calcium ions and activating magnesium ions. By this method a precipitate of calcium phosphate is formed in the vicinity of the enzyme, and this is rendered visible by conversion to black cobaltous sulphide.

A few experiments were made to ensure the validity of the technique in its application to the material used in this investigation. From each of four undegenerated sciatic nerves one slide was given the full Gomori technique, a second received no activating magnesium ions in the incubating mixture, and a third received no glycerophosphate, a fourth slide was incubated in the normal mixture with the addition of 0.1% potassium cyanide (known to inhibit alkaline phosphatase, Massart & Dufait, 1942), and a fifth slide was heated to 90° C. in water for 15 min., prior to normal treatment, in order to destroy the enzyme (Danielli, 1946). The sections on the first slide showed some blackening, those on the second showed less, and the remainder showed none. Similar experiments carried out on the other undegenerated nerves used in this study gave identical results. It is concluded that in peripheral nerve the technique reveals the activity of alkaline phosphatase as it has been shown to do in other tissues.

Control sections of nerve, omitting glycerophosphate from the substrate, were incubated as a routine in order to reveal any phosphate already present. Sections of kidney (known to be rich in phosphatase) were prepared together with those of the nerves in order to ensure that the technique was working satisfactorily. A further

check was provided by polymorphs in the blood vessels of the nerve, which appear intensely black after the phosphatase technique has been applied to them. Only sections in which polymorphs were intensely stained will be considered.

The location of phosphatase in relation to collagen was investigated by comparison of sections stained for the enzyme with sections fixed in 4 % formol-saline and stained by Gomori's (1937) silver-impregnation method for collagen and reticulin. For general histological comparison, sections fixed in Susa's fixative and treated with Masson's light-green method were used. For revealing axons in undegenerated and short-term degenerated nerves, Bodian's (1936) silver method was used.

RESULTS

Controls

Control sections of undegenerated nerves, and sections of nerves degenerated for all the periods of time employed in this work, did not in any case show any blackening when the Gomori technique with the omission of glycerophosphate was applied to them. Pre-existing phosphate was therefore negligible.

Undegenerated peroneal and tibial nerves

The greater part of the undegenerated nerve showed little positive reaction, but a number of sporadically distributed intense black 'streaks' were often visible in longitudinal paraffin sections (Pl 1, fig 1). When the number of 'streaks' in a section was large and their intensity of staining great, other structures in the nerve were often stained, particularly the precipitated myelin framework and the axons of myelinated fibres, and the intensity of staining of these structures was generally greater in regions in close proximity to the 'streaks'. In transverse sections (Pl 1 fig 2) the 'streaks' were apparently situated in interstices between the myelinated fibres.

The above description is based on eleven sciatic nerves. Considerable variation occurred, however, in the amount of blackening in these nerves. In some the 'streaks' were numerous, but in two nerves none was visible, though polymorphs in blood vessels were stained.

Other undegenerated nerves

The distribution of alkaline phosphatase activity in several other undegenerated nerves, differing in their distributions of fibre sizes, was investigated in an attempt to analyse further the curious distribution in the peroneal and tibial nerves.

Vagus, depressor branch. This nerve contains, like the peroneal nerve, a large number of heavily myelinated fibres. Like the latter, its alkaline phosphatase was distributed mainly in several 'streaks', with a few nuclei and some myelin framework also stained. (Three nerves.)

Greater splanchnic nerve. Most of the fibres of this nerve are finely myelinated, with a very small number of heavily myelinated fibres among them. There were a few 'streaks' of intense alkaline phosphatase activity, lesser activity in nuclei and axons of all myelinated fibres, and slight activity in myelin framework. (Three nerves.)

Anterior mesenteric nerve. This unmyelinated nerve was usually obtained with its

ganglion Throughout the nerve, nuclei were strongly stained and also fine fibres of uncertain nature (Pl 1, fig 3) In some of these nerves the intensity of staining diminished with increasing distance from the ganglion The ganglion cells showed intense phosphatase activity in the nucleoli and nuclear membrane, but not in the cytoplasm All the tissue surrounding the ganglion cells was very intensely stained (Pl 1, fig 4) (Three nerves)

Vagus, gastric branch The whole of this unmyelinated nerve showed a fairly intense phosphatase activity associated with all the nuclei, and with fine fibres, as in the anterior mesenteric nerves There was no localization in 'streaks' (Three nerves)

Degenerated sciatic nerve

The most proximal piece of the peripheral stump, comprising 2 or 3 mm of nerve, was affected by the trauma of operation, and is considered, together with any scar tissue which attached the end of the nerve to the adjacent muscle, as the 'traumatized' region Other pieces are referred to as the 'peripheral' region

Two days of degeneration, peripheral region The alkaline phosphatase reaction was similar to that of the undegenerated nerve Some frozen sections showed the 'streaks' particularly clearly Each 'streak' was fibrous, and contained blackened nuclei (Pl 2, fig 5) Some could be traced for 1 mm or more (Pl 2, fig 6) (Four nerves)

Traumatized region This was like the peripheral region, but myelin remains and axons were stained rather more at the traumatized end of the nerve, which was surrounded by many intensely staining polymorphs These had not invaded the nerve (Pl 2, fig 6) (Four nerves)

Five days of degeneration, peripheral region There had been little change since 2 days except that more nuclei showed a phosphatase reaction, and the surrounding cytoplasm sometimes showed a slight reaction also (Three nerves)

Traumatized region The cut end of the peripheral stump of the nerve was now securely bound to the adjacent muscle by connective tissue In silver-impregnated sections this was seen to consist of a tangle of fibres of all sizes which appeared to lie in a matrix Some were wavy purple ribbons, others were tangles of fine black threads, but most were very fine threads which were not intensely argyrophil When the alkaline phosphatase technique was applied to this connective tissue there was no blackening of any fibres Polymorphs had usually completely disappeared Just beyond the cut ends of about half the nerves of this group a few nuclei in the connective tissue were intensely stained for phosphatase (Eight nerves)

Ten days of degeneration, peripheral region A positive phosphatase reaction occurred in the great majority of nuclei of all kinds of cells, in certain fine longitudinally running fibrils (believed to be mainly Schwann cytoplasm) and in myelin debris In some nerves the distribution of phosphatase in longitudinal sections was streaky (Pl 2, fig 8) as at earlier stages, though the intensity of staining of the 'streaks' and their contrast with the rest of the nerve was not usually so great The 'streaks' consisted of groups of small Schwann tubes (devoid of myelin debris) in which numerous fibrils and nuclei were particularly strongly stained (Pl 2, fig 7) In most of the nerves there were no 'streaks' (Seven nerves)

Traumatized region The amount of connective tissue binding the end of the nerve to the muscle had increased, and in silver-impregnated sections many more collagen

fibres than at 5 days were visible, orientated rather more in a direction parallel with the nerve than otherwise. Blood vessels were easily distinguishable by their black reticular coats. There was no positive phosphatase reaction in the binding connective tissue except in the nuclei of blood-vessel walls and of polymorphs, and in some nerves, in a few nuclei just beyond the cut ends of the nerve fibres. The connective tissue fibres were completely negative (Pl 2, fig 8 and Pl 3, fig 9) (Seven nerves)

Fifteen days of degeneration, peripheral region Some streakiness of staining was still occasionally discernible. Almost all the Schwann, endoneurial and blood-vessel nuclei were strongly stained in most nerves (Pl 3, fig 10), and nuclei of perineurial cells were sometimes slightly stained. In some cells the cytoplasm of endoneurial cells could be seen to be feebly positive. In contrast to the 10-day nerves, stained myelin debris was almost entirely absent (Pl 3, fig 10). The blackened fibrils, present in the 10 day nerves, were more numerous, at any rate in the larger tubes, and often thicker. In transverse sections they appeared as rings of small dots against the inside of the neurilemma (i.e. the membrane lining the Schwann tube) and projecting into the lumen of the tube or sometimes apparently embedded in the substance of the neurilemma (Pl 3, fig 11). In longitudinal section some of the fibrils could be seen to end in perinuclear cytoplasm, identifying them as parts of Schwann cells (Pl 3, fig 10). In longitudinal sections stained with silver or Masson's light green there were rather more longitudinally orientated fibres present than at 10 days of degeneration, suggesting that new collagen had started to form. (Seven nerves)

Traumatized region Silver impregnation showed the connective tissue binding the end of the peripheral stump to the muscle to be deeply packed with thick, wavy parallel ribbons of collagen, all more or less orientated in the same direction as the nerve. This tissue showed little change in phosphatase reaction from the same region after 10 days of degeneration, though rather more nuclei of cells were stained, and their cytoplasm also showed a slight reaction. (Six nerves)

Twenty-five days of degeneration, peripheral region Signs of collagen formation in the form of an increased number of fine fibrils in the Schwann tube walls were quite obvious in silver-impregnated sections. The phosphatase reaction was little changed since 15 days of degeneration, except that the phosphatase-positive fibrils were thicker and more numerous. (Eight nerves)

Traumatized region This was similar to the 15-day traumatized region. (Seven nerves)

Fifty days of degeneration, peripheral region There had been a great increase in the number of longitudinally running collagen fibres seen in the walls of the Schwann tubes in silver-impregnated sections. In sections stained for phosphatase, nuclei were still intensely stained and there had been a further increase in thickness of the blackened fibrils seen in longitudinal and transverse section. (Nine nerves)

Traumatized region In silver-impregnated sections it was impossible to distinguish the original site of the cut, for the Schwann tubes were very collagenized and were continuous with the collagen in the connective tissue binding the stump of the nerve to the muscle. Near the cut end of the nerve partitions of phosphatase-positive material could be seen within some of the Schwann tubes. Strands of cells with nuclei and cytoplasm stained for phosphatase were found amongst the binding connective tissue, having probably migrated out from the cut end of the nerve, and some

of these strands appeared to have invaded the epineurium presumably by turning round and growing back along the nerve, as described by Masson (1932) (Eight nerves)

One hundred days of degeneration, peripheral region More extensive collagenization of the Schwann tube walls was obvious in silver-impregnated sections. In longitudinal sections stained for phosphatase nuclei were strongly positive, and very numerous fibrils were stained, some thicker ones intensely (Pl 3, fig 12). In transverse sections most of the staining other than that in nuclei was in the form of large lumps (fibrils seen end-wise) clearly situated within the lumina of the Schwann tubes. The main mass of new collagen, external to the neurilemma, was little stained (Pl 4, fig 13) (Three nerves)

Traumatized region In silver-impregnated sections no change other than more extensive collagenization was visible. Sections stained for phosphatase showed a very intense reaction in the strands of Schwann cells, which had wandered out from the cut ends of the Schwann tubes, and all surrounding tissue took up the stain to a certain extent (Pl 4, figs 14, 15) (Four nerves)

Tissue cultures of degenerated sciatic nerve

It was considered that tissue culture studies of degenerated sciatic nerve might help in elucidating the nature of the phosphatase-positive fibres seen in sections. Accordingly, pieces of degenerated nerve were removed for culture under sterile conditions at autopsy. Cultures of the peroneal branch of 15 and 25 days-degenerated sciatic nerves in hanging drops of fowl plasma and embryo extract showed good outwandering of Schwann cells, fibroblasts and macrophages. It was found that, if the clots were made as thin as possible and the cultures washed for half an hour in saline at 37° C prior to fixation in alcohol, the Gomori alkaline phosphatase technique could be successfully applied without the cells being obscured by blackening of the clot. The explant appeared intensely black, and all types of cells that had wandered out were stained, nuclei fairly intensely, cytoplasm less so. The Schwann cell cytoplasm appeared extremely fibrous, and it was possible to trace these phosphatase-positive fibrils into the explant and show that they were continuous with the phosphatase-positive fibrils of the nerve, thus indicating that the latter were cytoplasm of Schwann cells. Controls incubated without glycerophosphate were unstained.

DISCUSSION

Reliability of the histochemical technique

This has been reviewed by Danielli (1946) and Lison (1948). The specificity of the reaction appears to be good provided adequate controls are done for comparison. A positive reaction reveals the presence of the enzyme, but a negative result does not necessarily indicate its entire absence, since part of the enzyme activity may be destroyed during the processes of fixation, embedding, etc. Diffusion of intermediary substances in the section does not seem to occur, according to these authors, since the same result can be obtained by rendering visible either the phosphate or the alcoholic end of the ester substrate. A weak point of the technique may, according to Lison (1948), lie in the possibility that the enzyme diffuses during fixation from

the exact sites where it occurred under physiological conditions. Certain of my observations seem to support the view that diffusion, presumably of the enzyme itself, occurs. In the undegenerated sciatic nerve, nuclei and the myelin framework may be stained by the Gomori technique to an extent depending on the number and proximity of the intensely stained 'streaks'. In the 2-day degenerated nerve, these structures are more intensely stained toward the cut end of the nerve which bears a cap of intensely staining polymorphs. In the 100-day degenerated traumatized region, where the strands of Schwann cytoplasm that had wandered out sometimes show a very intense reaction, the surrounding connective tissue nuclei take up the stain to a certain extent, whereas, in sections of nerves where Schwann cytoplasm is not so intensely stained, connective tissue nuclei remain unstained. These and other observations suggest that, when a certain histological element is rich in phosphatase, some diffusion may occur, probably of the enzyme during fixation. The possibility of diffusion requires that no reliance should be placed on the finer points of localization, such as, for instance, the fact that all tubal and endoneurial nuclei, whatever their cell type, become phosphatase positive during degeneration. The existence of significant changes in distribution and activity of phosphatase during degeneration of the sciatic nerve is not called in question.

Alkaline phosphatase in undegenerated nerves

In the undegenerated sciatic nerves, except for two which were almost completely negative, phosphatase activity was chiefly localized in 'streaks'. These did not appear to involve the large fibres but to lie between them (Pl 1, fig 2). It was, however, difficult to decide from the undegenerated material what structures were stained in the 'streaks'. Very similar 'streaks' occurred during the early stages of degeneration (in some nerves up to 15 days), and here it seemed probable that they lay in the Schwann tubes formerly occupied by groups of small, perhaps unmyelinated, fibres. The hypothesis that the 'streaks' in undegenerated nerve represented groups of unmyelinated fibres and their associated Schwann cells cut somewhat obliquely led to the testing of other nerves. Those consisting mainly of unmyelinated fibres (gastric branch of the vagus, anterior mesenteric nerve) proved to be phosphatase-positive throughout. However, it must be admitted that this evidence is inconclusive since the fine details of the staining of these unmyelinated nerves were unlike the 'streaks' of the undegenerated sciatic, the unmyelinated nerves showing staining of numerous fine fibrils which were rarely distinguishable in the 'streaks'. An alternative hypothesis is, of course, that the 'streaks' are an artefact, due to diffusion and periodic precipitation of the enzyme during fixation, and peculiar to undegenerated nerves with large nerve fibres. On the basis of my data, however, no certain interpretation of the 'streaks' seems possible.

Alkaline phosphatase in the degenerated sciatic nerve

Between 5 and 10 days of degeneration the 'streaks' in the sciatic nerve disappeared, and from this time onwards phosphatase activity was uniform and increased progressively in amount, being chiefly associated with nuclei and with numerous fibrils. There is no doubt that many of these fibrils—probably all the intensely stained ones, consist of Schwann cell cytoplasm. This follows from their position within the

lumina of the Schwann tubes, which is especially evident after 50 or 100 days of degeneration. It is supported by the fact that in longitudinal section some fibrils could be traced up to the perinuclear cytoplasm of Schwann cells and by the tissue culture results. Remains of fine axons, although persisting up to 10, 15 and occasionally 25 days of degeneration, judging from Bodian preparations, can obviously be dismissed as a basis for the phosphatase-positive fibrils in the nerves that have degenerated longer. A certain number of the fibrils, especially those apparently embedded in the substance of the tube wall, and the numerous very fine, weakly positive fibrils of the long degenerated nerves, may well be connective tissue fibres.

Besides the predominant staining of all nuclei and of Schwann cytoplasm in the degenerated sciatic nerves, endoneurial cytoplasm is also often stained. In view of the possibility of diffusion of the enzyme, it may be that phosphatase reaction in some cells does not signify the original distribution of the enzyme. But it is nevertheless clear that during Wallerian degeneration there is a real onset, and subsequent intensification, of widespread phosphatase activity in cells which before degeneration contained no demonstrable phosphatase (except in the 'streaks'). This activity is not associated with the wave of mitosis which occurs between 4 and 25 days of degeneration and which results in the steep rise of cell population during that time (Abercrombie & Johnson, 1946*b*), nor with the destruction of nerve fibres which occurs during the same period.

Fell & Danielli (1943) tentatively suggested that alkaline phosphatase localized on newly formed collagen fibres, played a part in the collagen formation of rat and guinea-pig skin scars. Later observations have shown that an increase in histochemically demonstrable alkaline phosphatase activity commonly seems to succeed trauma to a tissue, for instance in skin (Fisher & Glick, 1947), bone (Bourne, 1948), and liver (Mellors & Sugiura, 1948, Sherlock & Walshe, 1947, Sulkin & Gardner, 1948), and collagen formation also commonly succeeds trauma. But can the hypothesis of Fell & Danielli be generalized in the light of the present results on degenerated nerves? In the peripheral stump of degenerated rabbit nerves the period of time during which a widespread cellular alkaline phosphatase reaction occurred certainly coincided with the peculiarly long-drawn-out phase of collagen formation found by Abercrombie & Johnson (1946*a*), and the onset of this phosphatase reaction coincided with the earliest appearance of new collagen according to my silver preparation (at about 10 days of degeneration). This temporal relation is, however, the only evidence I found in my material suggesting that alkaline phosphatase may take part in the process of collagen formation, and by itself it is inconclusive. The reaction was not particularly localized on new collagen fibres, so that rabbit nerve differs in this from the rat and guinea-pig skin studied by Fell & Danielli. Furthermore, in the scar region of severed rabbit nerve there was no demonstrable localization of the enzyme on newly formed collagen fibres. In the scar region indeed the phosphatase reaction, apart from the initial polymorph reaction, was not conspicuous anywhere during the early stages after the operation, although collagen fibres apparently formed during this period, and with very much the same time relations as after skin injury (as described by Hunt, 1941). Only after about 50 days of degeneration did considerable blackening appear, and then in the strands of Schwann cells which had wandered out from the cut ends of the nerve fibres. The conclusion must be that the relation between

histochemically demonstrable alkaline phosphatase and collagen formation differs in different tissues, and a generalization of Fell & Danielli's hypothesis of a causal relation between the two cannot therefore be supported by the available evidence

SUMMARY

1 Gomori's histochemical technique for localizing alkaline phosphatase activity has been applied to various undegenerated nerves, and also to the degenerating peripheral stump of the sciatic nerve of rabbits

2 Undegenerated nerves containing many myelinated fibres (sciatic, greater splanchnic and vagus depressor) showed phosphatase activity in sporadically distributed 'streaks', whose nature is discussed

3 Undegenerated nerves containing mainly non-myelinated fibres (anterior mesenteric and vagus gastric) showed a widespread reaction in nuclei and in fine fibrils of uncertain nature

4 In the degenerating peripheral stump of the cut sciatic nerve the 'streaks' had practically disappeared at the end of 10 days of degeneration, after which a reaction began to develop in nuclei of all kinds of cells and also in fibrils which mainly lined the Schwann tubes. Investigation of these fibrils by various methods, including tissue culture, indicated that most of them were cytoplasm of Schwann cells. They increased in size up to 100 days of degeneration at least, forming a thick, irregular lining to the neurilemma with intense phosphatase activity. The widespread enzyme activity in nuclei and cytoplasm coincides in time with the formation of collagen.

5 The scar tissue at the cut end of the nerve, which showed rapid formation of collagen comparable to that seen in healing skin wounds, revealed no significant alkaline phosphatase activity until after collagen had been extensively formed, the phosphatase was then associated with strands of Schwann cells that had wandered out from the end of the nerve.

I should like to express my thanks to Mr M. Abercrombie and Dr M. L. Johnson for suggesting this work and for their constant advice and encouragement during its progress. I am deeply grateful to Prof. G. R. de Beer for his supervision, and to Prof. J. L. Young for helpful criticism of the work. I am also indebted to Mr F. J. Pittock for taking the photographs. The work was done while holding a research studentship from the Medical Research Council.

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EXPLANATION OF PLATES

With the exception of Pl 2, fig 5 and 6, which were frozen sections, the material was fixed in alcohol embedded in paraffin and cut at a thickness of 7μ . Sections were then treated with Gomori's histochemical technique for demonstration of alkaline phosphatase. Linear magnification is given in brackets.

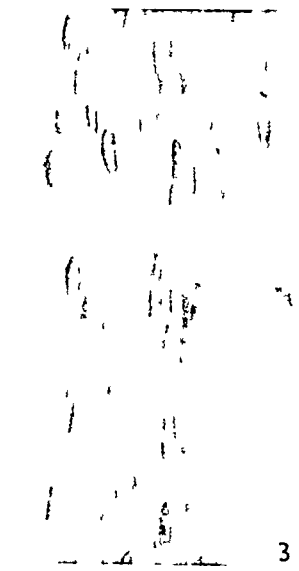
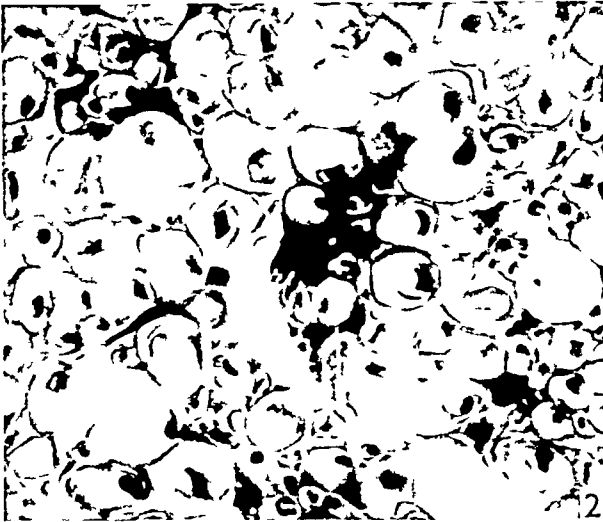
Abbreviations *m*, myelin debris, *n*, nerve stump, *s*, scar tissue, *S c*, Schwann cell cytoplasm.

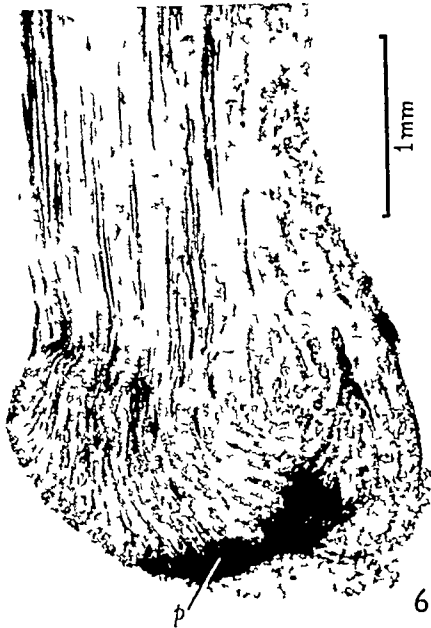
PLATE 1

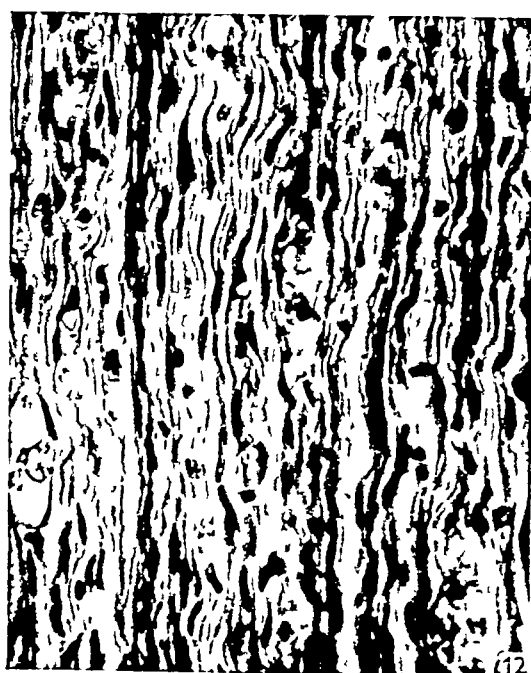
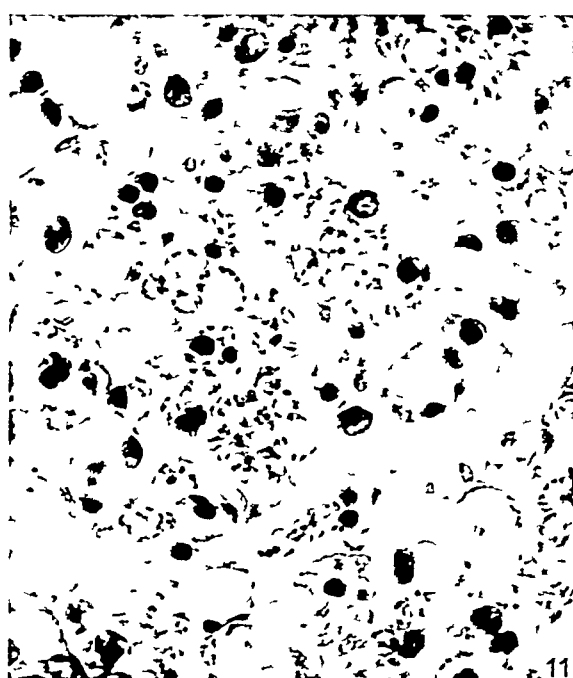
- Fig 1 Longitudinal section of undegenerated sciatic nerve (peroneal division) showing 'streaks'. Axons of large nerve fibres are also somewhat stained ($\times 105$)
- Fig 2 Transverse section of undegenerated sciatic nerve, showing 'streaks' cut across in interstices between large nerve fibres ($\times 615$)
- Fig 3 Longitudinal section of an undegenerated anterior mesenteric nerve, consisting mainly of unmyelinated fibres. Nuclei are stained, and also some fine fibrils which may be axons ($\times 680$)
- Fig 4 Section through an undegenerated anterior mesenteric ganglion. The ganglion cells show almost unstained cytoplasm, but the nuclei are darker with intensely stained nucleoli. Nuclei and fibres surrounding the ganglion cells are very intensely stained ($\times 645$)

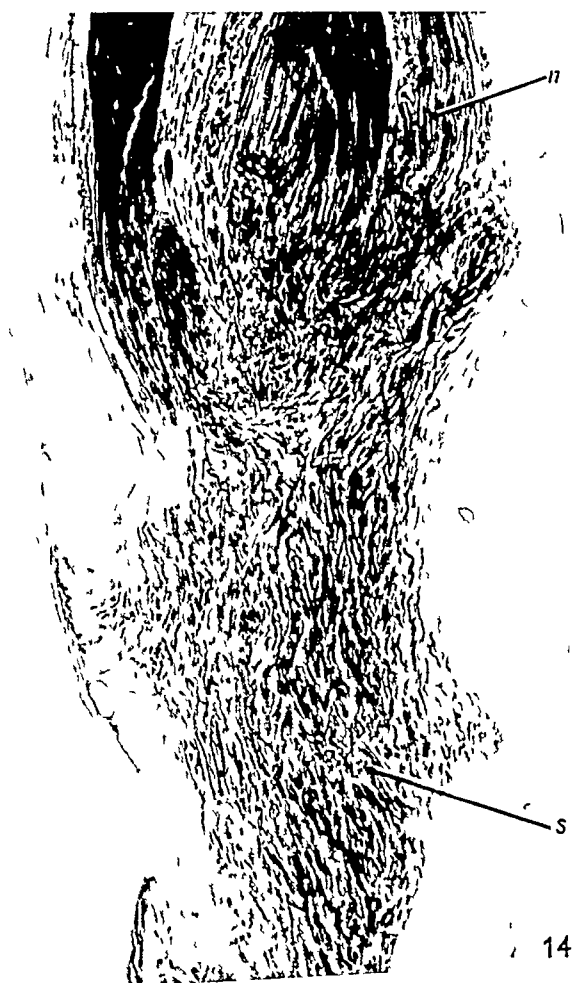
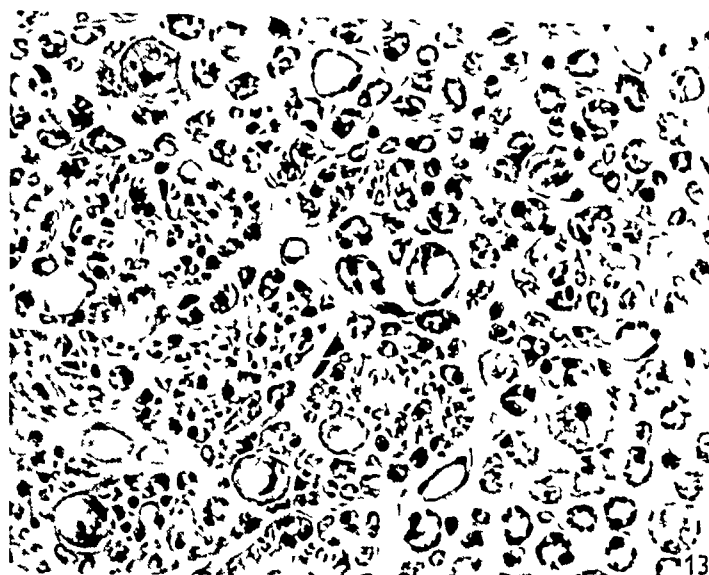
PLATE 2

- Fig 5 Part of a frozen longitudinal section of a 2 day degenerated sciatic nerve in the peripheral region showing 'streaks'. Nuclei in this neighbourhood are somewhat stained. Axons and myelin framework of myelinated fibres are slightly stained ($\times 330$)
- Fig 6 Frozen longitudinal section through the traumatized region of a 2 day degenerated peroneal branch of the sciatic nerve. A cap of intensely staining polymorphs (*p*) lies over the cut end ($\times 20$)
- Fig 7 Longitudinal section of a 10 day degenerated sciatic nerve, peripheral region. Nuclear staining is more widespread than previously, and fibrous strands believed to be Schwann cytoplasm are also stained. Myelin debris (*m*) is still stained, but has become somewhat disorganized. Axons are no longer distinguishable ($\times 355$)
- Fig 8 Longitudinal section through the traumatized region of a 10 day degenerated sciatic nerve. The polymorphs have disappeared and the scar region (*s*) shows hardly any alkaline phosphatase activity though collagen is rapidly being formed at this time ($\times 30$)









MARCHANT—ALKALINE PHOSPHATASE ACTIVITY IN THE NERVES OF THE RABBIT

PLATE 3

- Fig 9 Longitudinal section of scar tissue at the cut end of a 10 day degenerated sciatic nerve Two leucocytes in a capillary are strongly stained Otherwise the tissue is almost negative ($\times 405$)
- Fig 10 Longitudinal section of a 15 day degenerated sciatic nerve Nuclei and fine longitudinal fibrils (probably mainly Schwann cell cytoplasm, *sc*) stained Myelin debris no longer stained ($\times 455$)
- Fig 11 Transverse section of same nerve as in Fig 10 Black dots on Schwann tube walls are probably Schwann cells ($\times 675$)
- Fig 12 Longitudinal section of a 100 day degenerated sciatic nerve Fibrillar staining more strongly marked ($\times 445$)

PLATE 4

- Fig 13 Transverse section of same nerve as in Pl 3 fig 12 Black lumps within Schwann tube walls are probably Schwann cells Endoneurial cells are also stained ($\times 425$)
- Fig 14 Longitudinal section through traumatized region of a 100 day degenerated sciatic nerve There is intense staining of the Schwann cytoplasm, and the strands of Schwann cells (*s*), which have wandered out from the cut end of the nerve (*n*) can be distinguished from the surrounding connective tissue ($\times 18$)
- Fig 15 High magnification of scar tissue of the section shown in fig 14 There is intense phosphatase activity in the strands of Schwann cells, but surrounding connective tissue is scarcely stained ($\times 290$)

OBSERVATIONS ON THE FEMALE REPRODUCTIVE ORGANS OF THE CA'ING WHALE *GLOBIOCEPHALA* *MELAENA* TRAILL

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INTRODUCTION

The anatomy of the reproductive organs of the *Mystacoceti* has been described in detail by Mackintosh & Wheeler (1929), Ommanney (1932), and the literature has been reviewed by Mackintosh (1946). The cyclic changes occurring in the uterine mucosa of *Balaenopterid* whales have been the subject of a recent paper by Matthews (1948). His observations constitute the only detailed report, other than a brief reference by Mackintosh & Wheeler (1929), on the histology of the changes in the reproductive tract. Descriptions of the reproductive organs in the *Delphinidae* are, moreover, few in number, inconsistent, and give no reference to the histology of the ovaries or mucosa. Turner (1867) and Murie (1874) give a brief account of the immature vagina and uterus in *Globiocephala*. Meek (1918) describes the macroscopic appearances of the genital tract of *Phocaena communis*, now *P. phocaena* (four specimens), but there is little account of the ovary. The gross appearances of the genital organs of an immature *Delphinus delphis* are described by Pycraft (1932) and Khvatov (1938), quoted by Asdell (1946), who states that the corpora lutea are still present and show no sign of involution at the time of parturition. Comrie & Adam (1938) give an account of the female reproductive system of *Pseudorca crassidens*. They also describe the ovaries from fifteen females, giving particular attention to the corpora lutea, but making no histological observations.

The following observations on the female reproductive organs of *Globiocephala* are therefore presented to add to the scanty knowledge of a little described species. The histological appearances of the ovary, the corpus luteum and the mucosa of the uterus have not previously been described. Unfortunately, insufficient material does not permit of more than a partial comparison with the changes described by Matthews (1948) in *Balaenopterid* whales.

MATERIALS AND METHODS

The material to be described was obtained from a school of some 300 whales killed at Torshavn, in the Faroe Islands, in March 1947. Due to the kindness of Dr Carl Bech of Torshavn a representative selection of ovaries and genital tracts was removed from the females, and was at once fixed in 12% formalin.

The lengths of the majority of the whales from which the ovaries were taken are not known. However, Dr Bech states, in a personal communication, that the newborn young are 3-3½ ft in length, that immature specimens are of the order of 9-11 ft in length, and that adult females are up to 16 ft in length. The material obtained consists of the infra-umbilical part of one 42 cm foetus with the repro-

ductive system intact, the genital tracts of twelve females, mostly immature, and the ovaries of thirty-eight females. The size of the ovaries and three mean diameters of the corpora lutea and corpora albicantia are given in Table 1.

Representative portions were taken from the ovaries and, after sectioning, were variously stained with Harris's haematoxylin and eosin or van Gieson's stain, with Mallory's triple stain and by the methods described by Rossman (1942) for luteolipin. Portions of the uterine tube, uterine horns, fused horns, uterus and vagina were also sectioned and stained. It was found that immersion, prior to embedding, in 4% aqueous solution of phenol, with the addition of a little glycerine, facilitated the sectioning of the hard fibrous tissue without distorting the appearances.

THE REPRODUCTIVE ORGANS

The appearances of the external genitalia do not differ in any essential from the description given by Murie (1874).

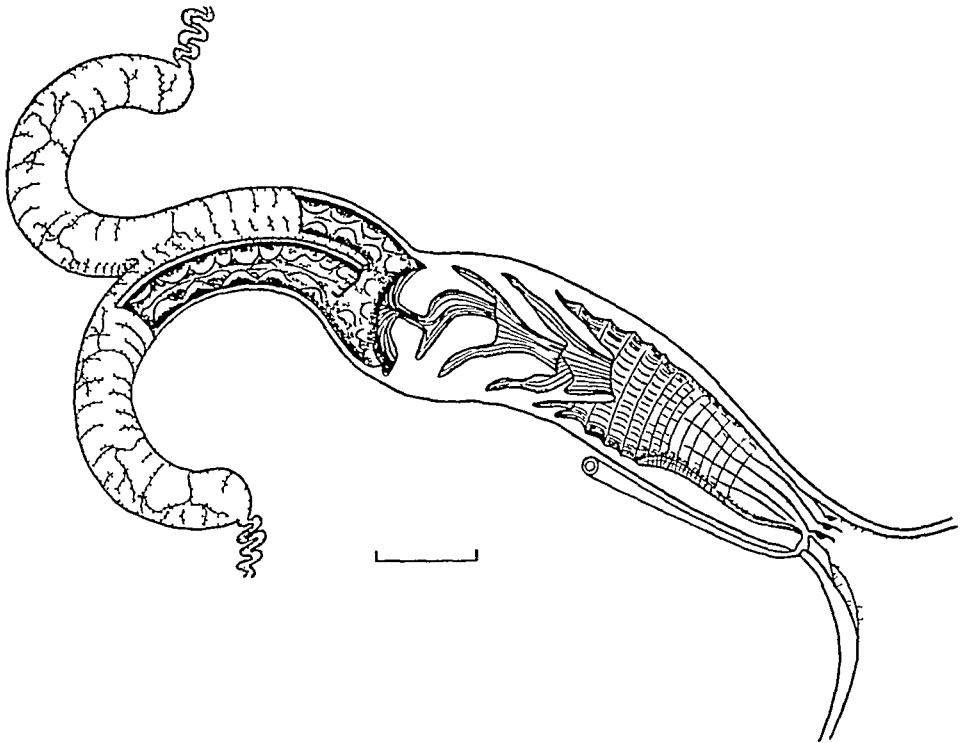
The female external generative aperture is an elliptical fissure near the middle of the third fourth of the animal's length, and inferiorly about vertical to the termination of the panniculus. The tegument around is dark coloured, and thrown into a good number of minute parallel and wavy wrinklings chiefly transverse in direction. Immediately within each tip of the vulva, or what may be considered the labia majora, are some twenty or more short, but deep, folds of membrane, in the recesses of which are crypts, the openings of sebaceous glands. The homologues of the nymphae or labia minora are two prominent folds of the mucous membrane, each one inch in length, which lie within the anterior pudendal commissure, and slightly converge as they pass backwards. Between these and with lateral plicae sulci is a median ridge 1.3 inches long, which ends in a small but distinctly pronounced clitoris.

In the 42 cm. foetus (Text-fig. 1) the wrinkling of the skin about the vulva is not present, and there are only six folds within the tip of the vulva, and their position is mainly lateral. The opening of the vulva is 2.0 cm. in length and 0.5 cm. in width, the junction of the vulva and vagina is markedly constricted. The vagina is 6.0 cm. in length, and it widens to 2.0 cm. in diameter at its upper portion, but narrows again at its upper limit. Immediately anterior to the opening of the urethra at the base of the clitoris lies a well-marked slightly raised area, in the form of a pear-shaped plaque 2 x 1 cm. on the ventral wall of the vagina. The surface of this plaque is only slightly grooved by shallow longitudinal fissures. In the maturing specimen the area is no longer raised in the form of a plaque and the vaginal wall in this lower portion is similar in its appearance to that of *Balaenoptera physalus* (Ommanney, 1932).

The structure of the upper part of the vagina is described by Murie as follows:

The upper half, on the contrary, is narrower and has a very uneven surface. This roughening depends on a numerous series of transverse rugae or puckerings of the membranes, some four of which are extremely prominent. In alluding to these valvular folds, Hunter (1787) aptly compares them to a succession of oesophageal rings. They are composed of thick induplications of the fibroid tissue of the wall of the vagina inwardly lined by narrow longitudinal mucous rugae, which fringe their free edges. The fold nearest to the os and only a thumb's breadth from it, has a thickness of 1/10 of an inch. The true os uteri is only distinguishable from the preceding folds by its narrower and somewhat firmer ring-like aperture.

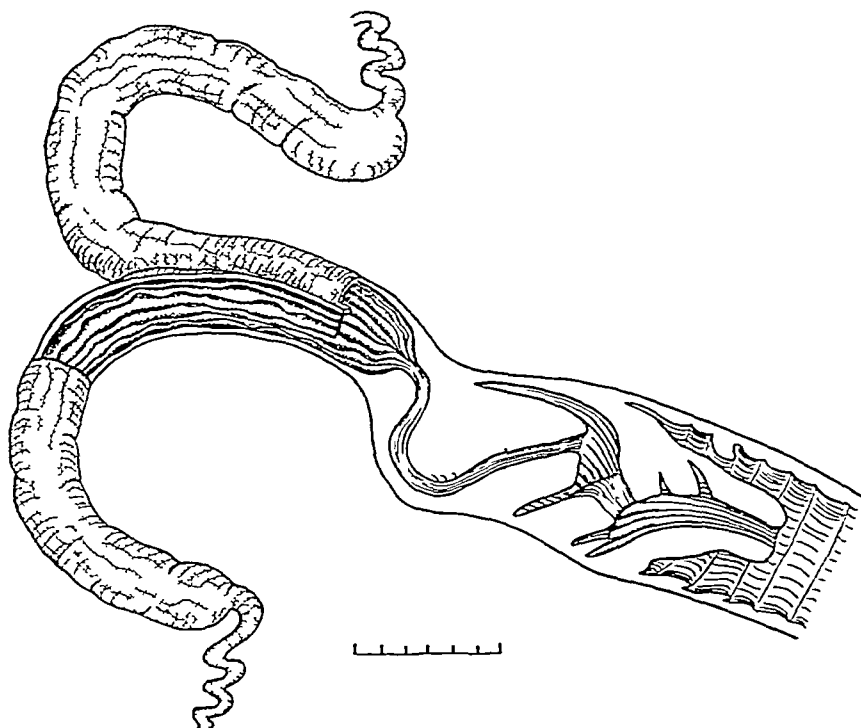
The appearances of the vagina in the 42 cm foetus and in the adult are shown in Text-figs 1 and 2. It will be seen that the uterine cavity opens through a small aperture into a narrow canal, each lined with mucous membrane that is thrown into numerous longitudinal folds. The canal has a curved course for about 8 cm in the adult and ends by opening through a large conical projection. It is considered that this canal represents the true cervical canal and that the conical projection is the cervix. Below the cervix there is a series of large transverse folds, two of which entirely encircle the vagina, and thus create two chambers. The cervix projects into the upper chamber, which leads into the lower through a narrow aperture. The lower chamber communicates with the lower part of the vagina through a further narrow opening in the second vaginal fold. The lower vaginal folds are drawn down



Text fig 1 Drawing of the reproductive tract of the 42 cm foetus. The vagina and cervix have been sagittally sectioned and parts of the posterior walls of the fused horns and left horn have been removed. The scale represents 1 cm.

into the vagina, so that they are similar in appearance to the cervix. The two main vaginal folds have secondary leaf-like folds arising from them. One such secondary fold is present in the 42 cm foetus (Text-fig 1), and a varying number up to four are found in the immature and mature specimens (Text-fig 2). All the folds are lined on their inner aspect by a series of parallel longitudinal rugae. Microscopical examination of the vagina and its folds, both in the foetus and in the adult shows them to be very muscular structures. A thin outer longitudinal muscle layer is present, which sends fasciculi up into the bases of the vaginal folds. The deep muscle layer is circular, well developed and arranged in a series of bundles separated by fibrous tissue. In the vaginal wall the bundles are small and numerous, but in the vaginal folds and cervix they are large and separated by much fibrous tissue.

The uterus is relatively small being 8 mm in length in the 42 cm foetus and 15 mm in the newly born specimens. The walls of the uterine cavity are smooth with a few raised protuberances or cotyledons. In the immature specimens the chamber measures 5.0 cm in length and in the adult it is 8 cm in length by 5.5 cm in breadth. In Murie's specimen an immature female the uterus measured 5 cm in length. The mucous membrane in the adult is raised into numerous longitudinal folds or rugae. These rugae are continuous with similar ones in the uterine horns and they narrow at the internal os and are continuous with the folds lining the cervical canal.



Text fig. 2. Drawing of the upper reproductive tract of the whale CW 11. The tract has been sagittally sectioned up to the level of the fused horns; one side of the fused horns and part of the left horn have been opened by removal of the posterior wall. The scale is in centimetre.

The uterine horns are fused for the lower 20 mm in the newly born and for the lower 10 cm of their extent in the adult. The free part of the uterine horn measures 9.0 cm in the newly born and 25 cm in the adult. The mucous membrane of the foetal horn is raised into a series of cotyledons and elongated rugae. However in the adult the mucous membrane is raised into a number of parallel longitudinal folds about 2 mm in width and 4 mm in depth. In places these folds are interrupted and it is apparent from the intermediate stages that the small cotyledons of the foetal and immature specimens become drawn out into the long parallel folds of the adult (Text figs. 1 and 2). The broad ligament is attached to the postero-lateral

aspect of the uterus and fused horns, the main bulk of the ligament passing behind the uterus. However, the ligament is attached to the lateral border of the separate uterine horns, and the first part of the uterine tube pursues its sinuous course in the substance of the broad ligament. Daudt (1898) describes a strong band running from the rostral end of the ovary to the foremost end of the ostium abdominale. Ommanney (1932) describes the same band in *B. physalus*. The band is also present in *Globocephala*, as also is a strong band running from the posterior aspect of the ovary to the uterine horn, considered by Daudt to be the ligamentum ovarii. The ligamentum teres is also present as a strong band running in the broad ligament from the base of the uterine horns to the inguinal canal.

In the material under discussion the uterine tube can be divided into three parts. The first part in the adult is sinuous and thick walled, and when drawn out extends for 6–10 cm. This leads into a wider, straighter, thin-walled part, which runs along the supero-medial aspect of the ovary to its anterior end. Here the tube opens out by a funnel-shaped pouch which overhangs the ovary (compare with the findings of Pycraft (1932) in the dolphin). The free lower and medial edge lies below the ovary so that the latter is virtually enclosed in a pouch, reminiscent of the ovarian sac of the seal. The inner aspects of the pouch and infundibulum are lined by numerous membranous ridges, which run downwards towards the ostium. These are presumably the fimbriae mentioned by Murie (1874). Microscopical examination shows that the inner aspect of the pouch is lined with small villous projections, covered with ciliated columnar epithelium and containing a narrow central core of delicate connective tissue.

Histological changes in the reproductive tract

Unfortunately there is insufficient material for an adequate report to be made on the changes occurring in the reproductive tract during the cycle, or during pregnancy. However, the complete tracts of two whales, CW 10 and CW 11, are available for examination. The first, CW 10, contained no corpora lutea in either ovary, and the presence of only a few small follicles less than 1 mm in diameter indicates that it is an adolescent specimen. The whale CW 11 contained the earliest corpus luteum of the series in one ovary, both ovaries displayed marked follicular development.

The mucosa, as Matthews (1948) describes in the Balaeopterid whales, is clearly divided into two layers. The outer subepithelial layer is composed of a thin stratum compactum, varying from 150 μ thick in CW 10 to 400 μ thick in CW 11, which contains the ducts of the glands and a superficial capillary plexus together with a few larger vessels. The deeper layer, the stratum spongiosum, is thicker, comprises the rest of the mucosa and contains the convoluted glands. The cells of the stratum compactum are relatively small, and tend to be stratified, giving the layer a distinctly more dense appearance than that of the stratum spongiosum. The cells of the latter layer are larger and are very loosely arranged. The epithelium has been retained over most of the mucosa, but in many areas, particularly of the mucosa of the body of the uterus, the epithelium has been lost. The writer agrees with Matthews (1948) that this loss of the epithelium is probably a post-mortem change occurring before fixation.

The uterus of the adolescent specimen CW 10 (Pl 1, figs 1 and 2) has its lining raised into a series of rugae 1-2 mm high and 1 mm thick similar, but larger elongated rugae are found in the fused horns and in the horns. The epithelium is low cuboidal or cubical, the nuclei are darkly stained and occupy most of the cell. The ducts are lined by high cubical or columnar epithelium and there is no visible lumen. The glands are straight with darkly staining nuclei and little cytoplasm, the lumen is small or not visible. The stroma is dense and compact all the cells stain heavily, there is no distinction of the stroma into two layers. The blood vessels are small and straight and there is a number of small vessels anastomosing just beneath the epithelium.

In CW 11 (Pl 1, figs 3-5) the lining of the uterus fused horns and horns shows distinct changes. The epithelium is of the high columnar pseudo-stratified type and the cytoplasm is increased in amount. The endometrium of the uterus is now 3-4 mm thick and the rugae are correspondingly enlarged in the horns. There is a distinct stratum compactum, containing the ducts of the glands, and numerous dilated anastomosing blood vessels. The stratum spongiosum is oedematous and the stromal cells are enlarged. The glands are hypertrophied, distended and coiled, and the basal part shows evidence of active proliferation. The lumen of each duct is dilated and active secretion is commencing in the glandular epithelium. The arteries are enlarged and tortuous, and the superficial vessels are engorged. In the superficial part of the stratum spongiosum the glands are larger in diameter, and the lumina are also larger. The glands in the mucosa of the uterus and the fused horns are larger in diameter than those of the horn. Small depressions can be seen in the surface of the mucosa, but because of the lack of material from later in pregnancy these cannot definitely be called incipient crypts, such as Matthews (1948) describes in Blue and Fin whales.

Transverse sections of the uterine tubes (Pl 2) show that the epithelium is of the pseudo-stratified columnar type. The epithelium of the tube of CW 10 is low cuboidal or columnar and the lumen is open and regularly stellate. In CW 11 the epithelium has changed to the high columnar type, the lumen is irregular and the epithelium more plicated. The epithelial cells are hypertrophied and the submucosa is oedematous.

It is therefore evident that the corpus luteum of CW 11, despite its small size and relatively early histological development, has initiated progestational changes in the reproductive tract.

THE OVARY

The ovary is suspended by a short thick mesovarium on the inner side of the broad ligament. In the foetus and the adolescent specimens the ovaries are white, elongated, oval bodies with smooth surfaces. There are no 'bramble' markings present, such as are found on the ovary of *Balaenoptera* (Ommanney, 1932). In the adult specimens containing a fully developed corpus luteum the shape of the ovary is almost spherical, due to the relatively greater mass of luteal tissue. The colour of the adult ovaries becomes a dark grey as the animal ages. The ovary is divided into cortex and medulla, the latter consisting of tortuous interlacing arteries and veins embedded in a dense mass of white fibrous tissue. The ovarian artery divides before

reaching the hilum into a large number of vessels, each accompanied by one or more veins. The main blood supply passes into the medulla but there are several small branches passing into the cortex. The cortex is relatively broad in extent and is filled with developing and atretic follicles embedded in the stroma. The tunica albuginea is thick, but it does not prevent protrusion of maturing follicles, or considerable herniation of the young corpus luteum. No interstitial cells have been observed in the medulla or the cortex.

In the hilum and proximal part of the medulla of the foetal ovary cords of poorly differentiated mesenchymatous cells are present. They probably represent the remnants of the rete ovarii. Remnants are also found in the ovaries of adolescent specimens. The cortex of the foetal ovary contains numerous primary oocytes, each oocyte being enclosed by a single layer of deeply staining flattened granulosa cells. The diameter of these primary oocytes is from 25 to 35 μ .

Large numbers of primary oocytes are found in the ovaries of the newborn and recently born specimens. Many of these oocytes show signs of impending or advanced degeneration. The ooplasm is distorted and vacuolated, and in many instances the oocyte has completely disappeared, leaving a flattened cavity surrounded by degenerating epithelial cells. There is no evidence of follicular growth in the youngest immature specimens.

The ovaries of the youngest specimens can be divided into three main groups from consideration of their size and volume, histological appearance and the size of the parts of the reproductive tract. The first group contains the ovaries of the newly born specimens (3) (ovary length 2.1–2.4 cm), the second consists of the ovaries of the immature specimens (4) (ovary length 2.6–3.2 cm), the third comprises the ovaries from adolescent specimens (7) (ovary length 3.8–4.5 cm). None of these ovaries contains a corpus luteum and only the last group contains developing follicles.

The ovaries from the third group of adolescent specimens (ovary length 3.8–4.5 cm), all show marked follicular development. Large numbers of small and medium-sized follicles are present and have a diameter varying from 0.5 to 6 mm. In two of the larger ovaries of this group large follicles are present, with a diameter of 2–2.5 cm. The largest follicle (fixed) seen in the whole series had a diameter of 3.25 cm and was in the ovary that contained the youngest corpus luteum (CW 11). The size of this follicle, when compared with that of the recently formed corpus luteum, indicates that the size of the mature follicle prior to ovulation is probably in the region of 3–4 cm.

Microscopical examination of the ovaries of this third group shows the presence of small numbers of healthy primary oocytes, 25 μ in diameter, in the cortex, there are also numbers of degenerating oocytes. No polynuclear oocytes or multiovarular follicles have been seen in any of the ovaries of the series. The structure of the maturing follicle is of the typical mammalian form, and there is marked development of the theca interna in the medium-sized follicles (Pl 3, fig 2). In many places developing follicles are surrounded by a theca interna layer some twelve cells thick. These cells show all the changes associated with glandular activity. The nuclei are enlarged and show vesicular changes, and vacuolation of the cytoplasm is a marked feature. Many developing follicles possess thecal cones (see Strassmann,

1941, Harrison, 1948) on the pole of the follicle nearest the ovarian surface. Some of the smaller follicles (0.3–1 mm in diameter) have internal cones on the pole of the follicle nearest the medulla of the ovary. The possible significance of the transient appearance of these inwardly directed cones of thecal cells has been discussed in a previous paper (Harrison, 1948).

Although there is insufficient material for a statistical analysis it appears that the growth of the ovum and follicle follows the usual two well-marked phases described (Parkes, 1931). The antrum develops when the follicle is 250–350 μ in diameter, and the zona pellucida appears at about the same stage, the oocyte being then of the order of 70 μ in diameter.

The granulosa cells in the healthy follicle show a basal layer of columnar cells, their nuclei arranged regularly with the long axis of each nucleus perpendicular to the lamina propria. The inner layers of the granulosa cells are cubical or spherical in shape and the nuclei are irregularly placed. Small spaces resembling the bodies of Call and Exner can be seen in the granulosa layer of maturing follicles (Pl. 3, fig. 2). Many of the follicles in the adolescent ovaries show signs of atresia. The even columnar arrangement of the basal layer of granulosa cells is disturbed, and this change coincides with the first appearance of degenerative changes in the oocyte. The granulosa cells lose their compact arrangement and the layer becomes loosened, the cells swell and subsequently degenerate. A further characteristic sign of early degeneration is a change in shape of the follicle. The peripheral pole of the follicle shows a tendency to collapse inwards, giving the follicle a pear-shaped appearance. The granulosa cells in the collapsed part of the follicle tend to remain undegenerated longer than those at the uncollapsed medullary pole. Thus collapse of the outer pole of the follicle has not been seen so markedly in ovaries of any other mammal examined, and it is apparently mainly limited to the ovaries of adolescent specimens. It is possible that it is due to the fact that the fibrous tissue of the medulla gives some stability to the inner pole of the follicle, whereas the actively growing stroma in the cortex, together with the presence of numerous follicles at various stages of development, exert a local pressure on the outer pole.

THE CORPUS LUTEUM

In the available material there are five ovaries which contain recently formed or fully developed corpora lutea (Pl. 3, fig. 1). Three of these corpora are from whales which had recently ovulated. Careful examination of the reproductive tract of the earliest of these specimens failed to reveal any products of conception. The details of the ovaries containing these corpora are given in Table 1.

The smallest of the corpora lutea (CW 11) has a diameter much less than that of a mature follicle, and there are no corpora lutea in the other ovary. There is considerable herniation of the corpus luteum, the herniated part being a nipple-like projection with a slit, 4 mm long at the apex of the projection. This slit, which is slightly depressed into the substance of the corpus luteum, marks the stigma. The cut surface of the corpus luteum is a pale yellowish grey in colour. The collapsed and folded mural epithelium is clearly seen, there is no marked haemorrhagic effusion into the centre (Pl. 3, fig. 1).

Microscopical examination shows a markedly folded epithelium. Considerable

trabeculation is present, projections of theca externa containing thecal vessels form the central core of the trabeculae (Pl 3, fig 3). A small slit-like central cavity is present, lined by fibroblasts. Large groups of theca interna cells are to be seen at the bases of the trabeculae and at the periphery of the developing gland. Some thecal cells have been carried into the developing gland about the trabeculae. The theca interna cells are relatively small, with a slight amount of cytoplasm containing several minute vacuoles, the nuclei are densely stained. The cells are smaller than

Table 1 *Measurements of ovaries and corpora lutea in adult specimens*

Animal no	Ovary				Corpora lutea* Mean of three diameters (mm)	Follicles
	Length (mm)	Breadth (mm)	Depth (mm)	Volume (c.c.)		
CW 11	50	30	25	15.5	18	Many small, several 1-3 cm
	55	30	20	16.0		
4	42	34	32	26.0	33	Many small, several 1 cm
	40	22	24	21.0		
16	80	25	15	24.0	20	None larger than 1 mm
12	65	22	23	16.0	18	Many small, several 1 cm
27	65	30	25	27.0	13	Many small, few 5 mm
	64	30	26	28.0	18	
19	55	25	24	20.0	12	Few less than 1 mm
21	50	30	20	13.0	7	Few less than 1 mm
1	60	60	40	88.0	50, 23	None
	55	30	28	22.0	17	
30	90	30	15	29.0	18, 17, 17	Few very small
25	65	25	25	24.0	16, 13, 9	Few very small
28	55	35	20	27.0	15, 9, 9, (? 1)	Many 5-10 mm
15	64	35	24	35.0	14, 12, 11, (? 2)	Few 5 mm
22	45	30	20	13.0	13, 12, 9, (? 2)	Few very small
	50	25	22	12.5	12, 8	
24	70	30	25	30.0	7, 5, 3, (? 3)	Few very small
3	60	40	20	32.0	20, 15, 14, 12	None
	55	35	20	32.0	17, 13	
23	55	30	20	18.0	13, 12, 10, 7	Two 3 mm, few very small
26	65	30	35	30.0	17, 14, 10, 7, 6	Few small
20	65	35	30	24.0	15, 12, 9, 8, 5, (? 1)	Few small, one 10 mm
18	55	25	18	16.0	12, 9, 7, 5, 4, (? 1)	Few small
2	70	50	30	64.0	30, 21, 18, 13, 10, (? 1)	None
5	70	60	35	66.0	41, 17, 11, 9, 7, (? 1)	None
	65	30	25	21.0	13, 12, 7, (? 2)	
29	50	30	18	24.0	13, 8, 8, 8†, 7, 6, (? 1)	Few very small

* The figures in brackets indicate the probable number of further corpora lutea, which are not visible to the naked eye, but can be seen in histological sections

† See p. 248

those found at the periphery of developing follicles. The granulosa cells show varying degrees of lutealization. Those cells near the periphery of the gland have an average diameter of 15μ compared with that of 10μ for the follicular granulosa cells. The majority of the cells have a granular eosinophilic cytoplasm, some cells already showing evidence of vacuolation. The vacuoles are either numerous, small and spherical, or single, large and oval. The nuclei of the granulosa cells are for the most part small and darkly stained, a few show vesicular changes. The granulosa cells are loosely arranged, being separated by fluid-filled spaces. There is some

fibrin between the cells and numbers of erythrocytes are also present in the fluid-filled spaces. Vascularization of the gland has commenced and small sinusoids and capillaries have been formed from the thecal vessels. Endothelial cells can be recognized invading the peripheral part of the gland. Early reticulum deposition has commenced in the peripheral part of the gland.

The general appearance of this early corpus luteum is therefore of an open 'lace-like' arrangement of lutealizing granulosa cells, such as has been described by Pearson & Enders (1943) in the fox, and by Harrison (1946) in the mare. It is apparent that ovulation has only recently occurred, approximately within the previous 48 hr, and from comparison with the appearances with those of other mammals, probably within 24 hr. The size of this corpus luteum, with a mean diameter of 19 mm, suggests that the size of the original mature follicle was of the order of 3-4 cm in diameter. Comparing this tentative size of the mature follicle with that of other follicles present in the ovary, it appears that in *Globocephala* only one or two follicles enlarge to a maximum diameter prior to ovulation.

The second and third specimens (CW 2 and CW 4, Table 1) are similar in appearance and structure, and the corpora lutea are approximately at the same stage of development. Both corpora lutea are markedly herniated, and the stigma is more depressed than in the previous specimen. A vertical slit passing through the centre of the gland, marks the remnants of the central cavity. The cut surface is yellower than the previous specimen, but the lobulated structure is still apparent (Pl. 3, fig. 1).

Microscopically the trabeculae have increased in thickness, mainly due to invasion by theca externa cells and increase in size of the vessels. The theca interna cells are still present as groups of small eosinophil cells, their cytoplasm devoid of vacuoles and their nuclei darkly stained. These groups are mainly arranged in the vicinity of the trabeculae, but some cells have wandered out between the luteal cells.

The granulosa cells have changed considerably and have increased in size to an average diameter of 30μ . The cytoplasm has increased greatly in amount and also in granularity. Some cells have an evenly granular cytoplasm, but others show varying degrees of vacuolation. In some cells the peripheral cytoplasm is full of small vacuoles, giving it a honey-comb appearance. The peri-nuclear cytoplasm is dense and granular. A striking characteristic of many luteal cells is the presence of two or more nuclei in one cell. These multinucleated cells are large, having a diameter of over 40μ (Pl. 4, fig. 1). Their nuclei are large and vesicular, but many of them are darkly staining, with the chromatin gathered into coarse granules. Occasional mitotic figures can be observed. There is little doubt that at this stage in the development of the corpus luteum there is mitotic division of the nuclei of the luteal cells. The cytoplasm of the cells does not necessarily divide, however, and, as a result, large multinucleated luteal cells are formed. The luteal cells tend to be arranged in columns, some four or more cells in breadth, in which many of the cells exhibit this multinucleated condition. The luteal columns are loosely arranged and are separated by fluid-filled spaces (Pl. 4, fig. 2). Endothelial cells are to be seen invading these spaces and vascularization of the corpus luteum is taking place. In many places, near the periphery, endothelial sinusoids have been formed around groups of luteal cells.

The fourth corpus luteum (CW 1) to be described is larger than the previous specimens and the cut surface has a pale ochre colour (Pl 3, fig 1) The gland is pear-shaped, markedly herniated, and a broad dimple marks the position of the stigma The trabeculae have further increased in size, giving the gland a lobulated appearance on section This corpus luteum is one of pregnancy, but the foetus was, unfortunately, not accurately measured in the field

Marked histological changes have occurred The trabeculae are thicker and consist of fibrous tissue, and the blood vessels in them have thick walls (Pl 4, fig 3) The luteal cells are collected into columns or groups by a network of fibrous tissue The vessels running in the fibrous network are flattened and contain little blood The luteal cells are smaller, having an average diameter of 25μ Multinucleated cells are still to be seen, but all the nuclei are smaller and darker staining than in the previous specimens Very few of the cells are vacuolated, and the cytoplasm is evenly granular in all the cells At the periphery of the gland small irregularly shaped cells with pale eosinophil cytoplasm and small darkly staining nuclei can be seen interspersed amongst the luteal cells It is presumed that these cells are the theca interna cells Such a statement cannot be verified without following the changes through a number of stages, but it is unlikely that such a series will be obtained

The last specimen (CW 5) is slightly smaller than the previous one and shows signs of impending retrogressive changes (Pl 3, fig 1) The specimen was obtained from a pregnant animal, but the length of the foetus is not known The fibrous matrix is even more pronounced and there is evidence of shrinkage of the luteal cells (Pl 4, fig 4) None of the luteal cells contain vacuoles and the cytoplasm has a 'fibrous' appearance Many of the nuclei are pyknotic or fragmenting There is little blood in the vessels of the gland, and the majority of the large vessels are developing thick walls Multinucleated cells are present, but are infrequent Theca interna cells can still be seen at the periphery of the gland and their appearances are the same as in the previous specimen

This corpus luteum is considered older than the previous specimen on account of the shrinkage of the luteal cells, and the signs of retrogressive changes

In the ovary of CW 29 a small yellow body 10 mm long, 8 mm wide and 5 mm in depth was seen Unfortunately the other ovary was not obtained This body was sectioned and was found to have a structure resembling an active corpus luteum The histological appearance is of a corpus luteum older than that of CW 1 and younger than that of CW 1 There is no trabeculation and little fibrous tissue in the gland Typical luteal cells are present, some containing small spherical vacuoles in the peripheral cytoplasm Multinucleated luteal cells can be seen and the nuclei are all vesicular Theca interna cells with their typical appearance are present at the periphery of the gland The structure is very vascular and the vessels are distended with blood The appearances thus indicate an apparently active area of luteal tissue It is noteworthy that in the same ovary there is a larger, though degenerated, corpus luteum of some previous cycle or pregnancy The ovary contains no follicles The lack of knowledge of the state of affairs in the other ovary prevents anything but a tentative suggestion as to the nature of this structure However, it is possible that it is an accessory corpus luteum, derived by the lutealization of a small follicle

THE RETROGRESSING CORPUS LUTEUM

It is well known that in the *Mystacoceti* the corpus luteum of each ovulation persists in a degenerated form for a long time and possibly throughout the life of the whale (Mackintosh & Wheeler, 1929, Mackintosh, 1946, for review) Comrie & Adam (1938) have investigated the retrogression of the corpora lutea of *Pseudorca crassidens*, and their results will be discussed in association with the findings in *Globiocephala*

In the material available eight ovaries contain one retrogressing corpus luteum, two contain two, seven contain three, four contain four, and four contain five, in addition to the five recently formed or fully developed corpora lutea already referred to. All the retrogressing corpora lutea are of different sizes, but their histological appearances are the same. Externally they are apparent as raised, puckered plaques on the surface of the ovary with a wrinkled ridge or ridges around the raised button-like area. The largest old corpus luteum has a mean diameter of 23 mm and the smallest discernible to the naked eye is 3 mm in mean diameter. Below this size old corpora lutea raise no wrinkles on the ovarian surface. Sections of the cortex of the ovaries containing many old corpora show the presence of further areas simulating the appearance of a degenerating corpus albicans. Therefore, although remnants of corpora albicantia remain visible for a long time in *Globiocephala*, it appears that such remnants shrink to a degree which does not enable them to be seen on the surface of the ovary. It is also to be noted that one of the ovaries (CW 24) which, by its dark colour and atrophic appearance, is from an old individual, contains visible remnants of only three old corpora lutea. These remnants are 7, 5 and 3 mm in mean diameter and have thus undergone retrogressive changes for a considerable period. However, microscopical examination of areas of the cortex revealed at least three further old corpora lutea of a mean diameter of 3, 2 and 2.5 mm, and which were invisible to the naked eye. It thus appears that retrogression in the corpus luteum and in the corpora albicantia is essentially similar to that occurring in other mammals, except that the retrogressive process extends over a greater length of time. The corpora albicantia eventually shrink to a size unappreciable by the naked eye, and they may presumably disappear entirely.

In one of the specimens (CW 30) three of the corpora albicantia are of approximately the same size and are arranged in a row, each being a pedunculated mass on a broad stalk.

DISCUSSION

An arrangement of folds in the upper part of the vagina resembling the condition in *Globiocephala* has been described by Pycraft (1932) in other species of Delphinidae, and this author gives the term 'pseudo cervix' to the elongated and conical appearance of the vaginal folds. He comments on the wide range of variation in the details of the anatomy of the uterus and vagina in the Delphinidae. Ommanney (1932) gives a selection from the accounts of various authors showing the variation in the number of the vaginal folds. They vary in number not only in the species, but also in the individuals of a single species. Daudt (1898) describes nine to twelve folds in *Phocaena phocaena*, whereas Meek (1918) finds only two great folds. Daudt (1898) finds twelve folds in *Balaenoptera physalus*, whereas Ommanney (1932) finds six folds. The appearances in *Globiocephala* suggest that there is a primary pattern of

two entire folds below the cervix, to which secondary folds are added, possibly during the growth of the whale

Murie (1874) describes four vaginal folds in *Globiocephala*, but one of these folds is apparently the external os of the cervix opened up. His illustration, in fact, shows the internal os, the cervical canal and the external os, and two complete vaginal folds. This interpretation shows an arrangement of the vaginal folds similar to that found in the specimens under discussion. Turner (1867) remarks, indeed, that the lips of the cervix are liable to be confounded with the upper transverse folds of the vagina.

Meek's suggestions (1918) that the penis probably penetrates the vaginal folds and that their function is to promote the emission of seminal fluid appear to be correct. It is also probable that they assist in conveying the seminal fluid towards the uterus by their muscular contractions. The seminal fluid is able to collect in the chambers formed by the vaginal folds, and their arrangement, and also the relative narrowness of the vulva will prevent sea water entering the uterus.

The killing of so many members of one school of *Globiocephala* at the same time enables some information to be obtained on the reproductive pattern. There are present in this one school on the same day at least one animal that has recently ovulated, two which have ovulated some days previously, two which are approximately in the middle of the gestation period and one full-term foetus (not obtained for this report). There are also seven recently born or immature females and nine adolescent females, the ovaries of the latter showing varying degrees of follicular development. The remaining ovaries contain several corpora albicantia visible to the naked eye and varying in number from one to five. Remnants of further corpora albicantia can be found on microscopical examination. It thus appears that there is no definite breeding season, although it is possible that ovulation does not occur during the winter months. However, so small a series does not completely rule out such a possibility. If it is assumed that the gestation period is from 9 months to a year (the gestation period of the Common Ocean Dolphin is said to be 276 days by Khvatov (1938) quoted by Asdell (1946)), the pregnant whales ovulated during the summer months of 1946, and the first group of newly born females was born in the early spring of 1947, and the adolescent females during the previous year. This estimate would suggest that the whale CW 11 was born in 1945 and has thus reached puberty at the end of its second year.

It is agreed with Comrie & Adam (1938) that the site of ovulation appears to be situated anywhere on the surface of the ovary. There is no tendency for the mature follicle to rupture at the poles of the ovary as in the mare (Harrison, 1946). Comrie & Adam note a greater retrogressive effect on the corpora albicantia in ovaries containing a corpus luteum of pregnancy. In the specimens of *Globiocephala* no such increase in the retrogressive process is noted. Corpora albicantia in the ovaries of CW 1 and CW 5 are of the same size as those in other ovaries from non-pregnant animals.

Comrie & Adam note a gradual gradation in the sizes of the corpora albicantia in *Pseudorca*, which they suggest indicates that the whale is polyoestrous. Although not as many corpora albicantia are present in one ovary as in *Pseudorca* there are similar regular gradations of the size of the corpora albicantia in *Globiocephala*.

In *Pseudorca* there are no corpora albicantia present of the same size, except at the final stages. However, there are in *Globiocephala* several instances of an ovary containing corpora albicantia of approximately the same size and appearance, as in CW 23, 28, 29, 30 (Table 1). The latter contains three quite large corpora albicantia, arranged in a row on short peduncles. This finding does not necessarily indicate polyovulation, as corpora lutea in this condition do not all retrogress at the same rate. Twinning and multiple pregnancies have been reported in the *Mystacoceti* (Asdell 1946), but in *Pseudorca*, only one foetus was found in each of seven specimens, and in *Globiocephala* only one foetus was found in each of three specimens. From this evidence and from the fact that the whales killed are at different stages in the reproductive cycle or pregnancy, it is probable that in *Globiocephala* as in *Pseudorca*, ovulation is spontaneous and occurs several times a year.

It is well known that the accumulations of corpora albicantia in the ovary of the whalebone whales is an indication of their age (Mackintosh, 1946). The methods of estimation are, however, based on the assumption that the corpora albicantia never completely disappear. It has been suggested from the observations given that in *Globiocephala* the corpora albicantia do eventually retrogress to such a degree that, failing serially sectioning each ovary, no precise indication of the number of corpora albicantia in an adult ovary can be given. A further criticism of any method of determining the age of a whale is that there is at present no infallible method of telling if any corpus albicans is one of the cycle or of pregnancy. Comrie & Adam suggest that the finding of a regular gradation in the size of a series of corpora albicantia indicates a polyoestrous cycle, it may, however, equally well indicate a successive series of pregnancies. Thus CW 5 which contained at least one corpus luteum of pregnancy and four corpora albicantia in one ovary, and five corpora albicantia in the other, is either 4-6 years old, depending on the number of oestrous cycles in one year, or 21 years old, assuming that each corpus albicans represents a corpus luteum of pregnancy and that the whale becomes pregnant every 2 years (see Mackintosh, 1946, Comrie & Adam, 1938). There still remains the unknown number of corpora albicantia that are invisible to the naked eye. For this reason counting the number of corpora lutea is no indication of the age of any particular specimen of *adult Globiocephala*.

The presence of multinuclear luteal cells in the early corpora lutea and also in those of pregnancy, has not hitherto been described in any other mammal. Multinuclear luteal cells have, however, been previously illustrated, though no reference is made to them in the text. In Mackintosh & Wheeler's description of the ovary in the Blue and Fin whales large vacuolated multinuclear cells from the corpus luteum are illustrated in fig. 125 on p. 388. There is growing evidence that the granulosa cells in many animals, probably including man, do divide mitotically in the first few hours after ovulation. Furthermore Bullough (1946) has suggested that the mitotic divisions occurring in the granulosa cells of the mouse just before and just after ovulation are due to the mitogenic effects of oestrogen. In this connexion it is interesting to note that the oestrogen value of the liquor folliculi of the Blue whale, *Sibbaldus musculus*, has been found to be of the order of 2000 m.u./l. by Jacobsen (1935). This figure may be compared with those of 92-788 m.u./kg. of fluid from the follicles of the cow given by Parkes & Bellerby (1926).

It is therefore possible that the formation of multinuclear cells is associated with the relatively high content of oestrogen in the follicular fluid. The incomplete division of the luteal cells may possibly be associated with the large amounts of lipids that the luteal cells contain. The multinuclear cells appear at the time that vacuoles are found in ever increasing amounts in the luteal cells. However, a more likely factor is the relative anoxia to which the cells are subjected. At the time when the multinuclear cells are found vascularization of the large quantity of luteal tissue is only poorly developed. Thus the multinuclear conditions may well be the result of an unrestrained growth process possibly associated with high oestrogen content occurring in a relatively anoxic environment. The presence of mitoses in the luteal cells in the retrogressing corpora lutea of *Sorex araneus* described by Brambell (1935) is probably due to some other factor, perhaps the removal of the stimulus (to the corpus luteum) of the L H from the anterior pituitary, or the relative increase in oestrogens derived from the placenta as pregnancy progresses.

SUMMARY

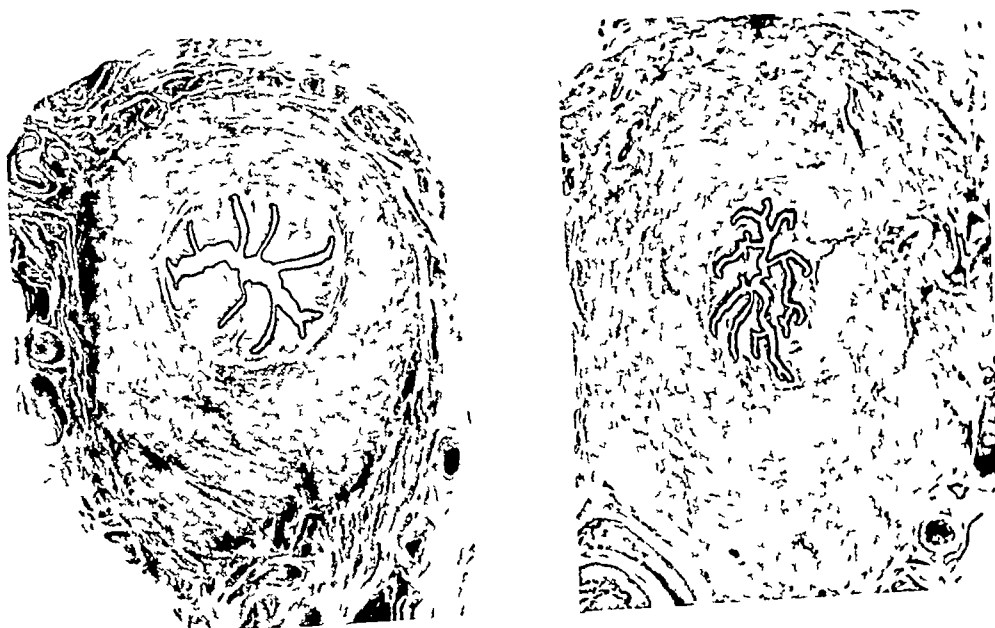
- 1 A general account is given of the anatomy of the reproductive tract in a foetus and in a number of adult specimens of *Globocephala melaena*.
- 2 The changes found in the ovaries are described, and a brief account of follicular development and atresia is given.
- 3 Five recent corpora lutea are described, multinuclear luteal cells are present in four of the corpora lutea.
- 4 The luteal cells are derived from the granulosa cells of the follicle, they become heavily vacuolated early in their development.
- 5 The theca interna cells mostly remain at the periphery of the developing corpus luteum.
- 6 Corpora albicantia persist in the ovary for a long time. However, there is evidence that they eventually shrink so that they are invisible to the naked eye.
- 7 The corpora albicantia are thus of little use in estimating the precise age of an individual adult *Globocephala*.
- 8 It is suggested that *G. melaena* is polyoestrous and that ovulation occurs spontaneously from one mature follicle at a time.

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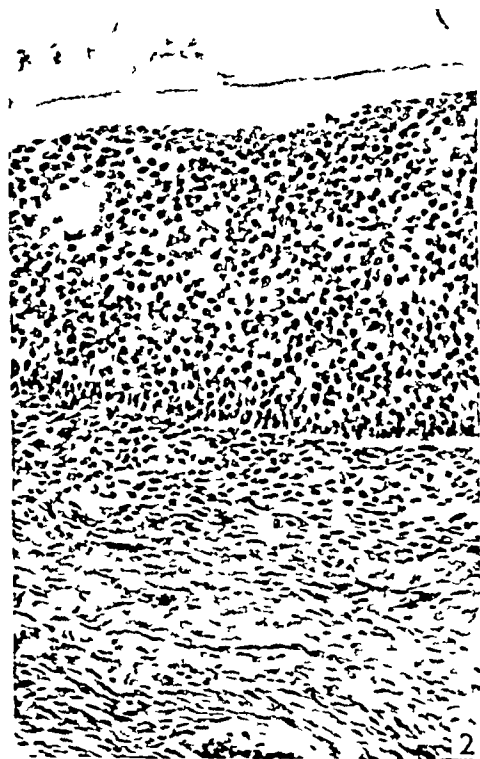
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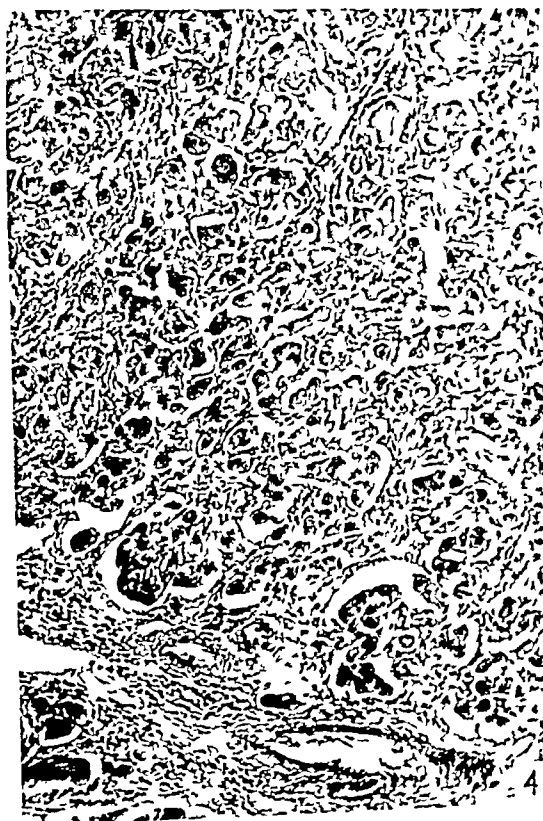
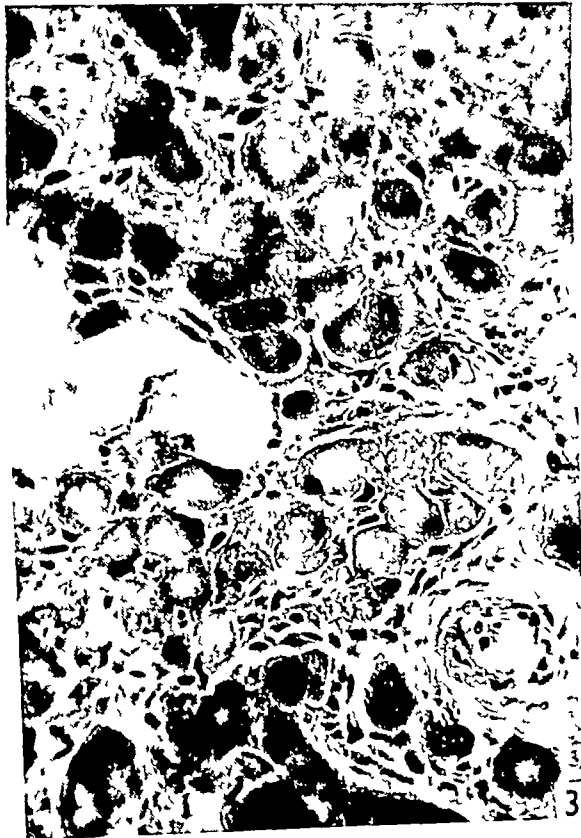
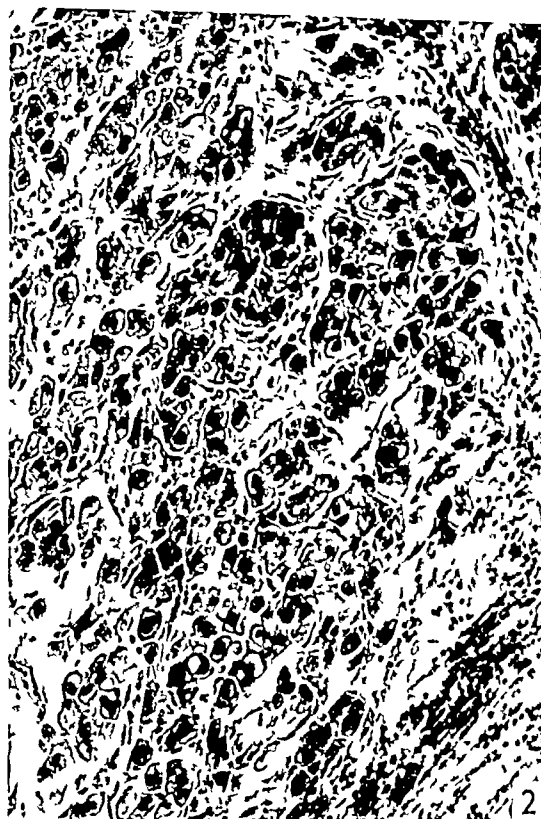
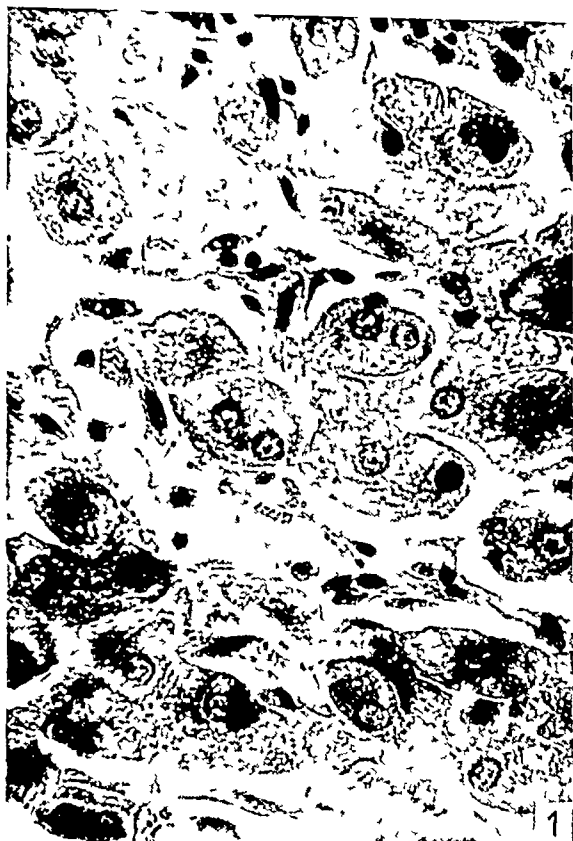
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EXPLANATION OF PLATES

PLATE 1

- Fig 1 Section through the body of the uterus of the anoestrous whale CW 10 Haematoxylin and eosin $\times 35$
- Fig 2 Section through the uterine horn of the anoestrous whale CW 10 Haematoxylin and eosin $\times 35$
- Fig 3 Section through the body of the uterus of the whale CW 11 An early corpus luteum is present in one ovary Haematoxylin and eosin $\times 20$
- Fig 4 Section through the fused uterine horns of the whale CW 11 Haematoxylin and eosin $\times 20$
- Fig 5 Section through the uterine horn of the whale CW 11 Haematoxylin and eosin $\times 20$

PLATE 2

- Photographs of sections through the uterine tubes of the whales CW 10 (left) and CW 11 The magnification of both sections is $\times 13$

PLATE 3

- Fig 1 Photograph of the macroscopic appearances of the five recent corpora lutea Scale in cm
- Fig 2 Section through the wall of a maturing follicle 1 cm in diameter The theca interna layer is clearly seen Haematoxylin and eosin $\times 80$
- Fig 3 Low power view of a section through the corpus luteum from whale CW 11 Haematoxylin and eosin $\times 30$

PLATE 4

- Fig 1 High power view of the luteal cells from the corpus luteum from whale CW 2 The multinuclear luteal cells can be seen Haematoxylin and eosin $\times 650$
- Fig 2 Section through the corpus luteum of CW 4 Haematoxylin and eosin $\times 120$
- Fig 3 Section through the corpus luteum of CW 1 Haematoxylin and eosin $\times 500$
- Fig 4 Section through the corpus luteum of CW 5 Haematoxylin and eosin $\times 90$

A PRELIMINARY STUDY OF THE DISTRIBUTION OF CELL SIZE IN THE LATERAL GENICULATE BODY

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INTRODUCTION

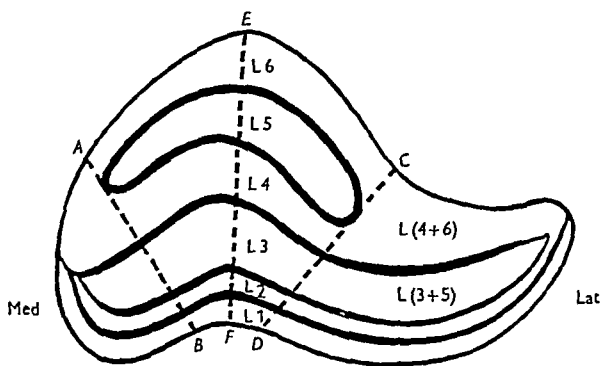
In the course of a comparative study of the laminar organization of the lateral geniculate body (Chacko, 1948*a*), a regular cell-size gradient was observed under the low-power microscope in the six-laminar central vision area as well as in the four-laminar peripheral vision area. It was decided to undertake a preliminary study of the spectrum of cell size, basing it on measurements of the size of the cells in the human geniculate nucleus in the various layers of both the central and peripheral vision projection areas. Supplementary data were obtained in a study of the same nucleus in the macaque monkey.

In studying the morphological details of the lateral geniculate body, it is apparent that the size characteristics of the cells lend themselves readily to quantitative measurements. The larger size and the more deeply staining character of the cells in layers 1 and 2 when compared with the cells in layers 3, 4, 5 and 6 have previously been recognized, and Balado & Franke (1937) have mentioned the approximate cell size in some of the layers. However, no systematic investigation of the mode of distribution of cell sizes has so far been undertaken. As regards the possible significance of such quantitative studies it should be recalled that Malone (1932) advanced the view that the size of a neuron is related to its specific function, to the volume of its activity, and to its tendency to maintain a uniform irritability. Although the central problem of cyto-architecture, namely, its functional significance, is far from solved, additional information along structural lines also may prove to be of value.

MATERIAL AND METHODS

A block of human brain tissue containing the lateral geniculate body was cut in 10 μ sections in a plane at right angles to the course of the optic tract and stained with Borell's methylene blue. The regions of the nucleus chosen for making measurements were selected in terms of the various retinal projection areas. In the central vision area which is, in general, characterized by six laminae, four antero-posterior levels were chosen at regular intervals from the foveal centre towards the periphery (cf. Sections A, B, C and D in Table 1). In the human brain, when these six laminae are traced to the peripheral vision area, layers 1 and 2 are continued as separate and discrete layers, whereas layer 3 fuses with layer 5, and layer 4 with layer 6 to form two 'small-celled' laminae (Text-fig. 1). These two composite layers of the peripheral vision area will be designated in the following as layers (3+5) and (4+6). In the projection area for the lower homonymous quadrants of the peripheral hemiretinae four antero-posterior levels were chosen, and in that for the upper homonymous quadrants three levels were chosen for measurements of the cells of their respective four laminae (cf. Table 1).

As was shown in a previous study (Chacko, 1948*a*), the central vision area has the shape of an inverted pyramid with a flattened apex directed towards the ventral hilum and a convex base directed towards the dorsal crest (Text-fig 1). In frontal sections this region appears roughly triangular, *EF* representing the axial plane corresponding to the horizontal meridian of the retina. The sample fields in each of the layers of the central vision area were located in a strip extending along the axial plane from the hilum to the dorsal crest. Since layers 1 and 2 (the large-celled elements) are very thin, less dense than the other layers, and limited in extent in this region, only 25–40 cells were found to be available for measurements in the sample fields of these laminae. In the case of layers 3, 4, 5 and 6 (the small-celled elements) 60 cells were measured in each layer of each of the four sections studied so that a total of 240 cells were measured in each layer, amounting to 960 cells in the whole central vision area.



Text fig 1 Diagram illustrating the extent of the central and peripheral vision projection areas in a frontal section through the human lateral geniculate body. *AB* indicates boundary between central vision area and the projection area for the upper homonymous quadrants of the peripheral hemi retinae (medial tubercle). *CD* indicates boundary between central vision area and the projection area for the lower homonymous quadrants of the peripheral hemi retinae (lateral horn). *EF* represents the axial plane corresponding to the horizontal meridian of the retina. *Lat* lateral *Med* medial.

The 'lateral horn', which represents the projection area for the lower homonymous quadrants of the hemi-retinae, exhibits a four laminar pattern throughout its extent. The sample fields in this region were located along a strip midway between the free margin of the 'lateral horn' and its junction with the central vision area. In the projection area for the upper homonymous quadrants, only the medial tubercle shows the typical four-laminar pattern and the measurements were, therefore, limited to the cells of the tubercle. Because this region is relatively limited in extent, in any single section, the strips chosen for measurements covered nearly the whole distance between the central vision area and the medial end of the tubercle. In both of the peripheral vision projection areas, the number of cells available for measurements in layers 1 and 2 was somewhat limited, although not to such an extent as in the central vision area. In most of the sections the number of cells measured represented the total number available in each section. The number of cells measured in each of layers 1 and 2 ranged from 18 to 47. In each of the composite layers

(3+5) and (4+6), 40-60 cells were measured. The monocular vision area was not included in the present study.

The central vision area of the geniculate nucleus of the macaque monkey was studied briefly in one typical section (Table 2).

In view of the fact that nerve cells may assume very irregular shapes, it is rather difficult to make precise measurements of their size. The cells of the human geniculate nucleus, as is evident from Pl. 1, appear triangular, polygonal, spindle- or flask-shaped. The method finally adopted for measurements consisted in measuring the long and the short diameters of the cell bodies by means of a screw eyepiece micrometer. The criterion on the basis of which cells were selected for measurements consisted in choosing only cells with intact and well-defined nucleoli. For further statistical studies the mean of the large and small diameters was noted for each cell.

RESULTS

The results of the measurements of the diameters of cells in various projection areas of the human geniculate nucleus are summarized in Table 1. First, the results obtained in measuring the cells of the central vision area will be considered. An inspection of the microphotograph in Pl. 1 shows not only the marked size differences between the cells of laminae 1 and 2 on the one hand and laminae 3-6 on the other, but also seems to indicate a size gradient from lamina 3 to 6. Such an impression is

Table 1 *Means and standard deviations of diameters of cells in μ measured in different laminae of various projection areas in the human lateral geniculate body*

Layer	No. of cells measured	Mean	σ
Central vision area			
Section A			
1	40	22.0 \pm 0.57	3.61 \pm 0.40
2	25	24.8 \pm 0.65	3.24 \pm 0.46
3	60	19.1 \pm 0.31	2.41 \pm 0.22
4	60	15.6 \pm 0.22	1.74 \pm 0.16
5	60	15.4 \pm 0.25	1.96 \pm 0.18
6	60	14.2 \pm 0.24	1.89 \pm 0.17
Section B			
1	30	23.9 \pm 0.38	2.09 \pm 0.27
2	28	24.5 \pm 0.61	3.25 \pm 0.43
3	60	18.9 \pm 0.27	2.12 \pm 0.19
4	60	17.8 \pm 0.31	2.37 \pm 0.22
5	60	15.8 \pm 0.20	1.54 \pm 0.14
6	60	13.8 \pm 0.25	1.95 \pm 0.18
Section C			
1	30	22.8 \pm 0.53	2.89 \pm 0.37
2	30	23.0 \pm 0.57	3.11 \pm 0.40
3	60	18.2 \pm 0.34	2.65 \pm 0.24
4	60	17.0 \pm 0.20	1.56 \pm 0.14
5	60	15.5 \pm 0.29	2.28 \pm 0.21
6	60	14.8 \pm 0.21	1.60 \pm 0.15
Section D			
1	30	23.6 \pm 0.62	3.40 \pm 0.44
2	30	23.8 \pm 0.58	3.19 \pm 0.41
3	60	19.5 \pm 0.25	1.96 \pm 0.18
4	60	17.9 \pm 0.29	2.22 \pm 0.20
5	60	16.9 \pm 0.31	2.40 \pm 0.22
6	60	14.7 \pm 0.24	1.87 \pm 0.17

Table 1 (continued)

Layer	No. of cells measured	Mean	σ
Projection area for the lower homonymous quadrants of the peripheral hemi retinae			
Section A			
1	30	21.7 \pm 0.54	2.98 \pm 0.38
2	18	22.8 \pm 0.61	2.62 \pm 0.44
(3 + 5)	60	17.5 \pm 0.22	1.74 \pm 0.16
(4 + 6)	60	16.8 \pm 0.22	1.72 \pm 0.16
Section B			
1	47	21.3 \pm 0.47	3.26 \pm 0.34
2	29	23.5 \pm 0.57	3.07 \pm 0.40
(3 + 5)	60	18.3 \pm 0.28	2.18 \pm 0.20
(4 + 6)	60	15.8 \pm 0.20	1.53 \pm 0.14
Section C			
1	44	20.4 \pm 0.40	2.65 \pm 0.28
2	32	21.2 \pm 0.55	3.12 \pm 0.39
(3 + 5)	60	17.8 \pm 0.24	1.89 \pm 0.17
(4 + 6)	60	15.4 \pm 0.19	1.52 \pm 0.14
Section D			
1	22	23.6 \pm 0.73	3.43 \pm 0.73
2	38	23.5 \pm 0.39	2.39 \pm 0.27
(3 + 5)	60	18.9 \pm 0.28	2.17 \pm 0.20
(4 + 6)	60	16.2 \pm 0.24	1.88 \pm 0.17
Projection area for the upper homonymous quadrants of the peripheral hemi retinae			
Section A			
1	20	23.6 \pm 0.42	1.92 \pm 0.30
2	20	21.4 \pm 0.51	2.27 \pm 0.36
(3 + 5)	40	16.7 \pm 0.32	2.01 \pm 0.22
(4 + 6)	60	15.5 \pm 0.22	1.67 \pm 0.15
Section B			
1	47	23.4 \pm 0.47	2.99 \pm 0.33
2	29	23.7 \pm 0.54	2.43 \pm 0.38
(3 + 5)	60	17.9 \pm 0.28	2.15 \pm 0.20
(4 + 6)	60	16.1 \pm 0.25	1.92 \pm 0.17
Section C			
1	40	23.0 \pm 0.41	2.60 \pm 0.29
2	20	25.8 \pm 0.37	1.64 \pm 0.26
(3 + 5)	60	17.0 \pm 0.21	1.62 \pm 0.15
(4 + 6)	60	16.8 \pm 0.29	2.27 \pm 0.21

confirmed by the data presented in Table 1. It is seen that there is a decrease in the mean diameters of the cells in the ventro-dorsal direction from lamina 3 to 6 in all the sections studied. Further analysis indicates that the differences between the means of the cell diameters in the small-celled laminae 3-6 are, in practically all instances, statistically significant. The fact that the cells decrease significantly in size in the ventro-dorsal direction is also evident from the histograms of laminae 3-6 in Text-fig. 2 and from the smoothed frequency polygons in Text-fig. 4.

As regards the results obtained in measuring the cells of the projection areas for the lower and upper homonymous quadrants of the peripheral hemi-retinae, the findings are similar to those in the central vision area in that there exists a size gradient in the small-celled layers (3+5) and (4+6). There is a decrease in the mean diameters of the cells in the ventro-dorsal direction from one lamina to the other (Table 1). The mean diameter of the cells in lamina (4+6) is significantly smaller than that of the cells in lamina (3+5) in six out of the seven sections studied. The histograms in Text-fig. 3 and the smoothed frequency polygons in Text-fig. 5 illustrate this decrease in size with reference to the projection area for the lower homonymous quadrants of the peripheral hemi-retinae.

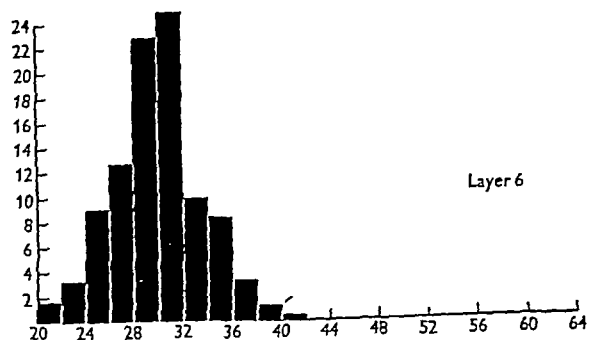
An analysis of the measurements in the sample fields of the large-celled laminae 1 and 2 does not reveal a size gradient as definite as that found in the small-celled laminae. As regards the central vision area, the mean diameter of the cells in lamina 2 is larger than that of the cells in lamina 1, but only in one of the four sections studied is the difference between the means statistically significant (Table 1, cf also Text-figs 2 and 4). In considering the measurements in the projection area for the lower homonymous quadrants of the peripheral hemi-retinae it is evident from Table 1 that the mean diameter of the cells in lamina 2 is larger than that of the cells in lamina 1 in three of the four sections studied. This difference is found to be at least tolerably significant (cf also Text-figs 3 and 5). In the projection area for the upper homonymous quadrants of the peripheral hemi-retinae the mean diameter of the cells in lamina 2 is found to be larger than that of the cells in lamina 1 in two of the three sections studied, but in only one is the difference statistically significant. It must be remembered that the laminar organization of this region is not as uniform as in the projection area for the lower homonymous quadrants, and that the medial tubercle presents a limited and rather unsatisfactory area for making measurements. If the data on cell diameters in the large-celled laminae of the central and peripheral vision areas are viewed as a whole, it becomes apparent that they have failed to establish any clear-cut size gradient.

In considering the transition from the large-celled to the small-celled layers, that is, the difference between laminae 2 and 3 in the central vision area and that between 2 and (3+5) in the peripheral vision areas, it is evident that the mean diameter of the cells in lamina 2 tends to be markedly larger in all the sections studied (Table 1). In all instances the differences between the means of laminae 2 and 3 or between 2 and (3+5) are statistically significant.

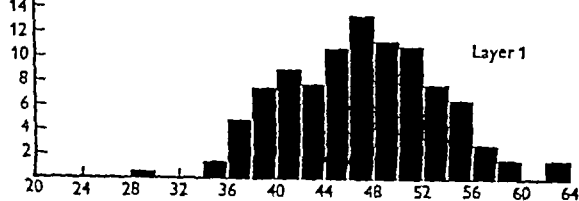
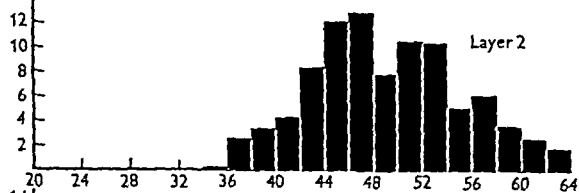
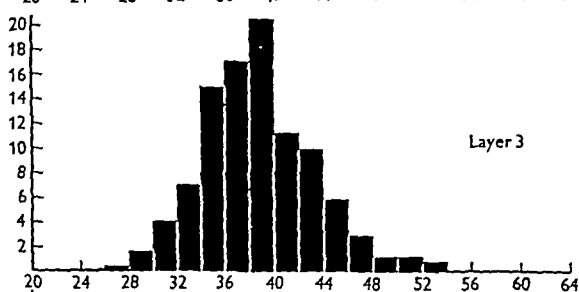
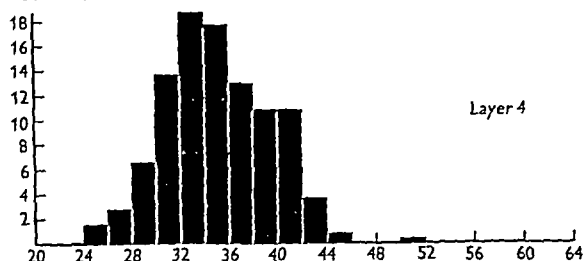
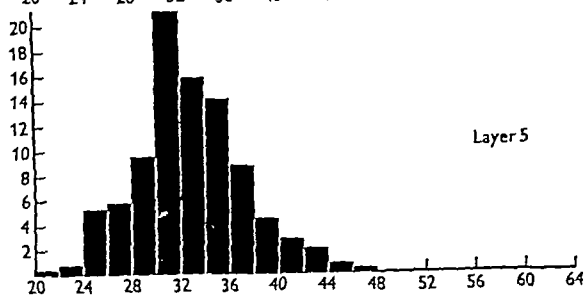
An inspection of the histograms in Text-figs 2 and 3 and of the smoothed frequency polygons in Text-figs 4 and 5 clearly indicates that the several laminae differ in variability or dispersion of measurements. The range of cell diameters measured in the dorsal laminae is definitely smaller than that in the ventral laminae.

An analysis of the results with reference to crossed and uncrossed laminae readily shows the significant differences which exist in the central vision area between the mean diameters of the cells in layers 1 and 4 and layers 4 and 6 as well as between the mean diameters of the cells in layers 2 and 3 and layers 3 and 5. Similarly, significant differences exist between the means of the crossed and uncrossed layers of the peripheral vision area, that is, between layers 1 and (4+6) as well as between layers 2 and (3+5). Since in Text-figs 4 and 5 the frequency distribution curves for the crossed laminae have been indicated by solid lines in contrast to the dotted lines for the uncrossed laminae, it can be seen at a glance that the curves for the crossed laminae are displaced towards the shorter diameters. This displacement is found in both central and peripheral vision areas and is especially marked in the small-celled layers. In Text-fig 4, although there is an overlapping of the curves, L6 is located more to the left than L5, L4 more to the left than L3, and L1 more to the left than L2. In Text-fig 5, L(4+6) is located more to the left than L(3+5), and L1 more to the left than L2.

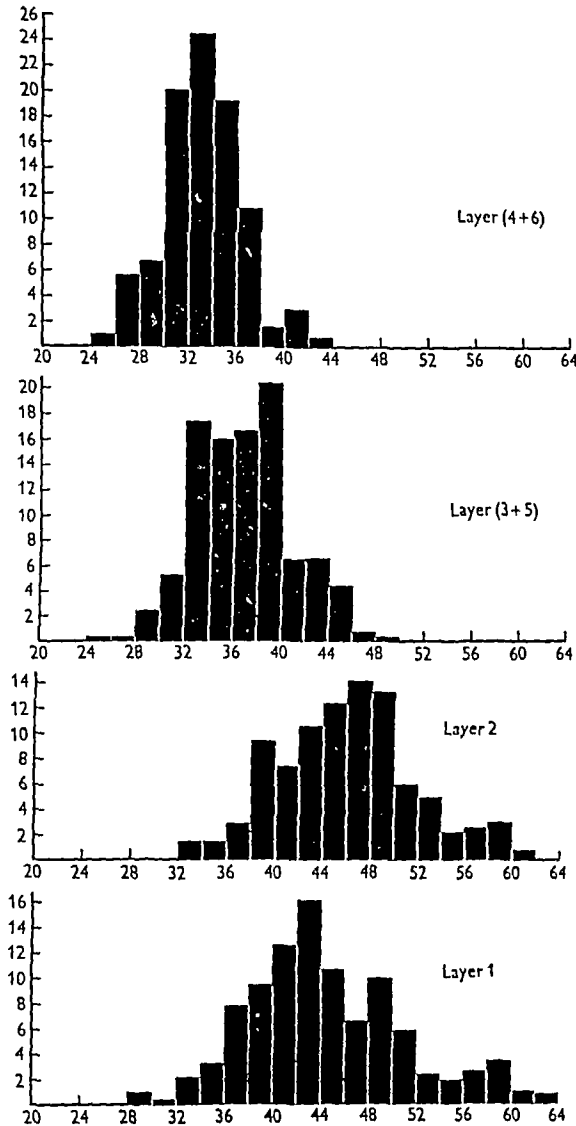
If the means of the cell diameters of the various laminae of the central and peripheral vision areas are compared (Table 1), it is found that the laminae with the



Text-fig 2 Frequency of diameters of cells in laminae 1-6 in central vision area. Abscissa diameter in $\mu (\times 2)$. Ordinate percentage frequency.



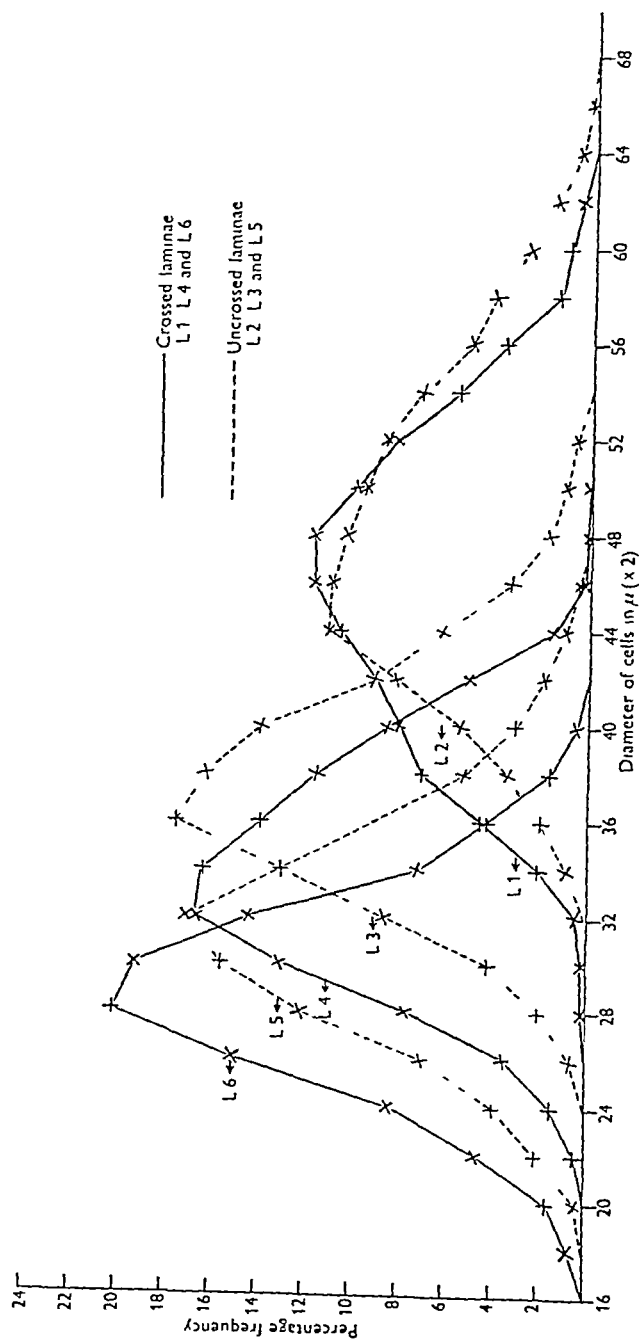
smallest mean cell diameters are located in the central vision area. There is a greater range of cell diameters in the central than in the peripheral vision area. This greater range is brought about by the occurrence of smaller cell diameters. Further, if the composite laminae of the peripheral vision area and their daughter laminae in the central vision area into which the former split, are compared, the laminae of the



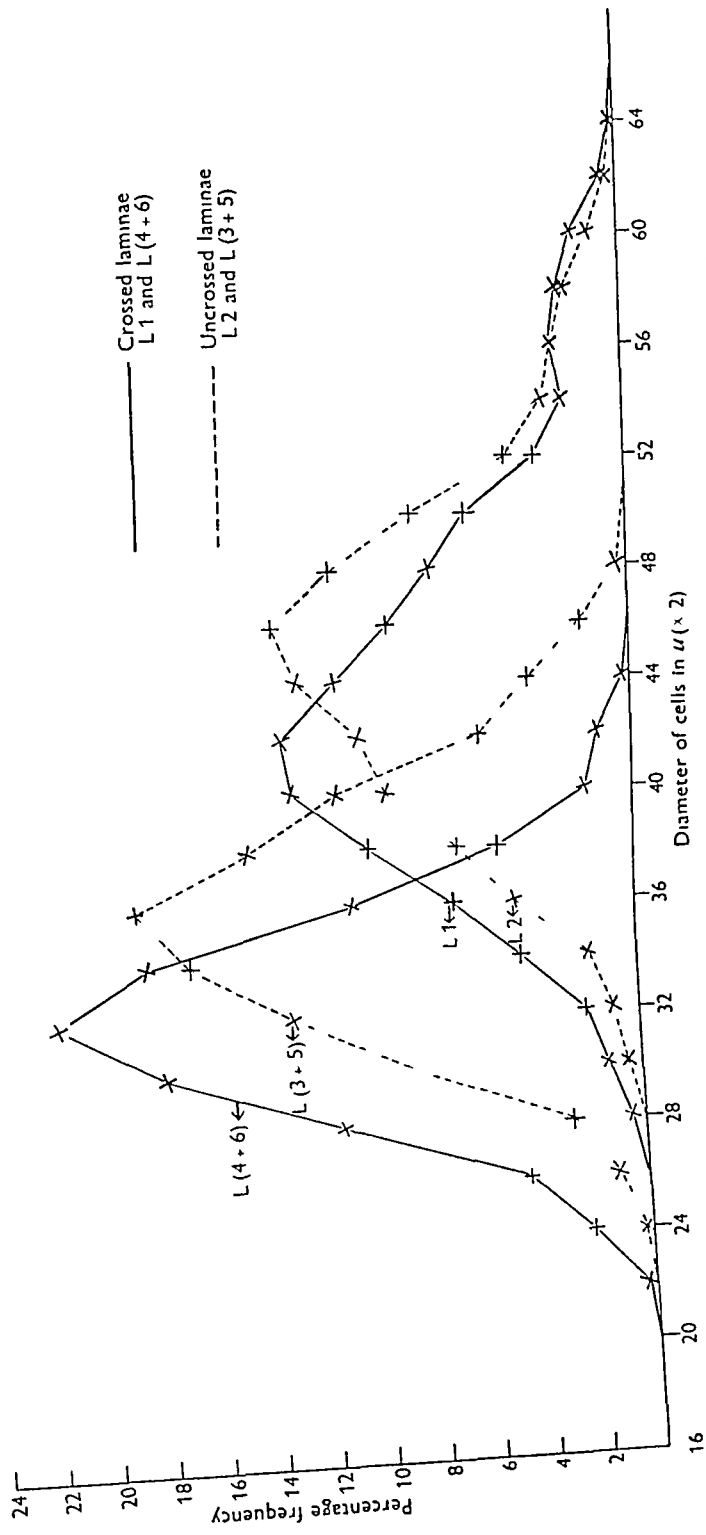
Text fig 3 Frequency of diameters of cells in laminae 1, 2, (3+5) and (4+6) in the projection area for the lower homonymous quadrants of the peripheral hemi retinae. Abscissa diameter in $\mu (\times 2)$. Ordinate percentage frequency.

central vision area are found to have a greater range of cell diameters. The mean of the distribution of all cell diameters measured in each of the parent laminae of both the peripheral vision areas lies between the means of the two daughter laminae. Similarly, the median of the distribution of the cell diameters of each parent lamina lies between the medians of the distributions of the corresponding daughter laminae.

From a comparative point of view it is of special interest that a cell-size gradient



Text fig. 4 Frequency of diameters of cells in different laminae of the central vision area



Text fig 5 Frequency of diameters of cells in the different laminae of the projection area for the lower homonymous quadrants of the peripheral hemi retinae

similar to that in the human geniculate nucleus is also present in the lateral geniculate body of the rhesus monkey (Pl 2 and Table 2)

Table 2 *Means and standard deviations of diameters of cells in μ measured in different laminae of the central vision area in the lateral geniculate body of the rhesus monkey*

Layer	No of cells measured	Mean	σ
1	28	23.5 \pm 0.47	2.47 \pm 0.33
2	28	24.5 \pm 0.65	3.20 \pm 0.43
3	28	17.6 \pm 0.40	2.12 \pm 0.28
4	28	15.3 \pm 0.36	1.90 \pm 0.25
5	28	14.9 \pm 0.30	1.57 \pm 0.21
6	28	14.5 \pm 0.37	1.97 \pm 0.26

DISCUSSION

It has been suggested by several investigators, but principally by Malone (1932), that the functional specificity of nerve cells is related to their internal structure, their shape, and their size. Malone has made an extensive study of the fundamental types of nerve cell structure. He emphasizes the point that the cell bodies and dendrites have a higher metabolic rate and exhibit a greater variation in histological character than axons, and assumes that these features are associated with a wide range of local activity radically different from that of the axons. According to him, the size of the cell body and dendrites is dependent on the activity of the neuron and the extent to which it receives stimuli. He points out, for instance, that typical correlating neurons have small cell bodies with extensively branched axons and poorly developed dendrites and, furthermore, that they are numerous and lie close together in centres in which impulses diffuse widely and do not enter into definite common paths in contrast to co-ordinating centres containing relatively few neurons with large cell bodies and well-developed dendrites. In his opinion, the resistance to fatigue varies with the size of the cell body, the small cell bodies of typical correlating centres fatiguing more easily than large cell bodies.

The present study has established the existence of a size gradient in the central and peripheral vision areas of the lateral geniculate body. The measurements indicate that the range of cell diameters is smaller in the peripheral than in the central vision area. It has also been found that each of the layers (3+5) and (4+6), when traced to the central vision area, splits into two laminae and that the means (as well as the medians) of the frequency distributions of the two daughter laminae are larger or smaller than the mean (or median) of the parent lamina.

In speculating on the possible significance of these findings, the attempt may be made to interpret the activity of the laminae as follows: a lamina possesses a number of neurons comprising a certain range of sizes and yielding a typical frequency distribution. These elements acting as a whole are capable of producing a certain type of total activity. The resultant activity of any particular lamina will be chiefly determined by the activity of its middle range elements, but such activity will probably be modulated by the elements at the two extremes of the distribution. The splitting of a particular lamina into two discrete laminae will widen the range

of activity within which each daughter bundle may then be assumed to establish its own specific maximum activity, and the latter is directly determined by its individual nerve elements. Various findings in sensory physiology and psychology clearly indicate that there exist numerous special functions with respect to which the discriminative capacities of the central retina are superior to those of the peripheral retina.

It has been previously pointed out in Part I, pp. 1 and 2 that the distribution curves for the crossed bundles L1, L4 and L6 are displaced towards the shorter distances when compared with the distribution curves for the uncrossed bundles L2, L3 and L5. Differently expressed, the cells of the uncrossed bundles are on the whole relatively larger than those of the crossed bundles. The bundles related to the corresponding halves of the two eyes are identical in number, but they are not necessarily duplicate mechanisms in functional respects since they are not identical morphologically. It appears likely, therefore, that the neural elements related to one eye respond to a certain extent differently from those related to the other eye whenever corresponding points of the retinae are simultaneously stimulated. The impulses from the two corresponding points are relayed to the same 'unit' in the geniculate body although it must be remembered that the crossed bundles of such a 'unit' are, as experimentation experimentally indicates, morphologically independent of the uncrossed bundles. Anatomical considerations of this nature thus lead to the assumption of certain functional differences between temporal and nasal halves of the retina, but they do not suggest that such differences are very pronounced. It is to be expected that the functional efficiency of either the temporal or nasal half of one retina is less than that of two homonymous hemi retinae and that the impulses arriving from the contralateral eye feed quantitatively and qualitatively to a widening of the response activity. The functional superiority of the nasal retina in visual acuity, colour sensitivity, etc., has been commented on by various investigators (Köllner, 1920; Hood, 1914; Cobb & Chubb, 1925; Krieger, 1927). It is Köllner's thesis that there exists an inequality of 'corresponding impressions' and a superiority of the nasal retina. The data of the present investigation do not suggest a functional superiority of either the nasal or temporal half of the retina, but they do seem to provide an anatomical basis for an inequality of 'corresponding impressions'. Since the nasal as well as the temporal retina, each in its own specific way, contributes to binocular vision, the conducting units of fibres from both eyes and the total number of bundles in each projection area will have to be taken into consideration in analysing 'units' in the visual system.

The existence of a size gradient in the central and peripheral vision areas of the lateral geniculate body is of particular interest in view of the existence of various morphological gradients in other parts of the visual system. In the receptor layer of the retina, the rods slowly and gradually increase in thickness and decrease in length from the central area to the periphery. Similarly, the cones change from short and thick to long and thin structures, thus exhibiting a gradient in regard to both size and shape. An investigation of the different varieties of bipolar and ganglion cells likewise yields size gradients. It has been observed that the axons and dendrites of the larger retinal neurones are thicker than those of the smaller neurones (Polak, 1911). A study of the optic nerve reveals a continuous size

spectrum (Chacko, 1948*b*) As regards the optic radiation, Polyak mentions that the calibre of the fibres varies from fine to medium and coarse fibres

If the assumption is made that cell size is one of the morphological characteristics related to functional specificity, cell-size gradients, such as exist in the geniculate body as well as in various layers of the retina, may be thought to have some relation to functional gradients of various kinds Gasser and his collaborators have, for instance, established an approximately linear relationship between the velocity of conduction and the diameter of nerve axons (Gasser, 1941) As regards the visual system, it mediates responses to differences in radiation varying in luminous intensity or in wave-length The responses made are frequently responses to stimuli of a graded character The organism must be able to fall back on mechanisms capable of dealing with stimuli lying in the same 'dimension', such as neutral colours constituting a brightness series or the variations in hue in certain portions of the spectrum The question may be raised whether cell size gradients or fibre gradients are related to or suggestive of mechanisms which cope with variations in the 'quantity' or 'quality' of light The problem also arises whether mechanisms, such as Granit's modulators (1947) yielding narrow sensitivity curves in different regions of the spectrum, have some counterpart in the anatomical organization of the lateral geniculate body Neither the histological nor the electrophysiological analysis has reached the point at which profitable hypotheses can be offered In the meantime, Hartridge (1948) is undoubtedly right in pointing out that Granit's modulators form 'what amounts to a polychromatic series' Further research will be necessary to determine whether the serial character of the modulators is in some way related to size gradients or other morphological gradients in the visual sector of the central nervous system

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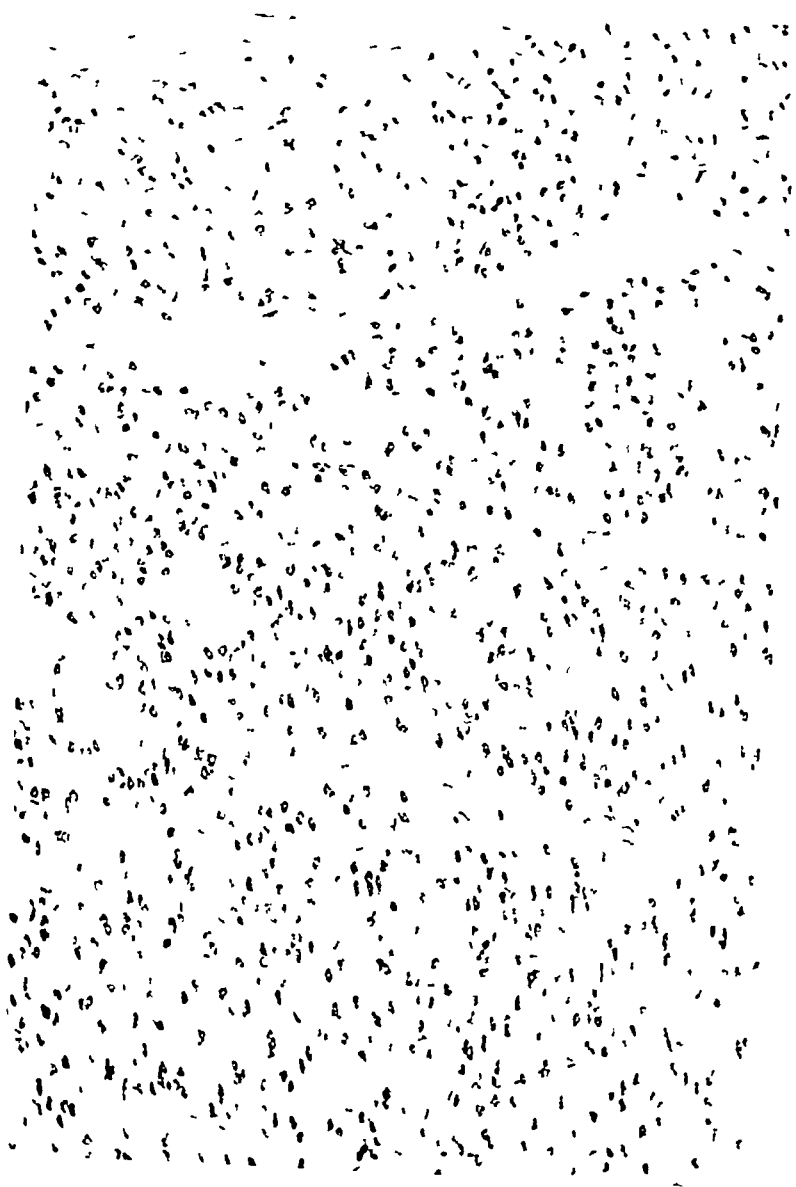
EXPLANATION OF PLATES

PLATE 1

Microphotograph of a section through the central vision area of the human lateral geniculate body $\times 80$

PLATE 2

Microphotograph of a section through the small celled laminae of the central vision area in the lateral geniculate body of the rhesus monkey $\times 80$



CHACKO—DISTRIBUTION OF CELL SIZE IN THE LATERAL GENICULATE BODY

THE DISTRIBUTION OF THE VASAL AND CREMASTERIC ARTERIES TO THE TESTIS AND THEIR FUNCTIONAL IMPORTANCE

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In a previous study of the vascularization of the human testis (Harrison & Barclay, 1948) attention was called to the distribution of the vasa (deferential) and cremasteric arteries, and the manner of their anastomoses with the testicular artery, and to the importance of these in relation to deliberate or inadvertent ligation of the testicular artery in surgical operations such as orchiopey, herniorrhaphy and radical operation for varicocoele. Although many observers have noted the effects of ligation or division of the testicular artery in experimental animals, it has not been possible to find any reference in the literature to work considering these effects in relation to the site of the anastomoses which the testicular artery makes with the vasa and cremasteric arteries. The present communication is concerned first with an experimental study of the effects on the rat testis of interruption of the testicular artery at definite points before and after its anastomosis with the vasa and, secondly, the manner of anastomosis of the vasa and cremasteric arteries with the testicular artery in Man as demonstrated by arteriography. Finally, consideration is given to the application of the experimental results to the problems of ligation of the testicular artery in Man.

EXPERIMENTAL RESULTS OF LIGATION OF THE TESTICULAR ARTERY

Historical resume

Cooper (1830) observed that division of the testicular artery and vein in a dog was followed by gangrene of the testis. Mallet (1879) embolized the testicular artery, or ligated the testicular artery alone, or the testicular artery and veins, or merely the testicular veins, in the dog. He observed the effects on the testis for periods up to 6 months, and found that any obstruction to the blood flow through the testicular artery led to a haemorrhagic infarct of the testis, as early as 48 hr. after interruption of the blood flow to the testis the cell content of the seminiferous tubules had coalesced to an almost homogeneous mass, and the interstitial tissue showed degenerative changes. Mallet concluded that the testicular artery has for the testis the significance of an end-artery in Cohnheim's sense. Enderlen (1896) claimed that a ligation left around the spermatic cord in the dog for periods up to 16 hr. caused no damage to the testis, periods longer than this led to atrophic changes. Griffiths (1896) found that after ligation of the testicular artery alone in dogs the testis showed great decrease in size in a few days, due to rapid destruction from degenerative changes in the seminiferous tubules, at later periods, particularly in one testis 3 months after operation, he found normal seminiferous tubules, which he concluded to be in process

of regeneration. Ligature of the testicular veins alone caused haemorrhagic infarction of the testis, ligature of the testicular artery and veins together eventually led to complete atrophy of the testis, except in one adult dog 130 days after operation in which the ligature could not be found and the testicular artery had established free communication again. Griffiths assumed from these results that the germinal epithelium can recover from a temporary degeneration and resume spermatogenesis. In experiments performed also on dogs, Capuro (1902) claimed that there are profuse anastomoses between the testicular and vasal arteries (fundamental arteries) and the cremasteric and external and internal pudendal arteries (accessory arteries). Even if all of these arteries are ligated on one side he claimed that an injection mass could pass to the testis by way of anastomoses with the other side. Schinz & Slotopolsky (1924), in their monograph on the experimental pathology of the testis, describe several experiments made on guinea-pigs and rabbits in which the testicular vessels were divided or compressed by scar tissue high up in the abdominal cavity following operations for the extirpation of the lumbar sympathetic chain, or when these vessels were directly ligated or sectioned, hypoplasia, 'regressive metamorphosis', necrosis or atrophy always resulted. They warn against the interpretation of results following ligature and not section of the testicular vessels. Section of the testicular artery only, according to Wangenstein (1927), is always followed by diminution in size of the dog's testis and scattered degenerative changes in the tubules, spermatogenesis still continuing. Ligature of the testicular artery and all the veins in the pampiniform plexus always results in total destruction of the testis, the latter becoming transformed into fibrous tissue. Ligature for 10 hr, and subsequent release, of all the structures in the spermatic cord of dogs and rabbits produces complete degeneration and fibrosis after 45 days, according to Iwasita (1939), this degeneration does not occur after only 5 hr compression.

In most of the above investigations no definite indication is given as to the exact point of ligature, or section of the testicular vessels, in relation to the anastomoses which the artery makes with other vessels. However, most observers are in agreement in finding that ligature of the testicular artery and vein is followed by degenerative changes in the testis. The present investigation is concerned with an examination of the effects of interruption of the testicular artery in the rat in specific relation to the site of anastomoses which this artery makes with the vasal artery.

The vascular pattern of the rat's testis compared with that of the dog

Before undertaking an experimental study of the effects of interruption of the blood supply of the testis in the rat, it was necessary to examine by means of arteriography the vascular pattern and anastomotic connexions in the normal animal. A parallel study was made of the vascularization of the dog's testis for the reason that, as already indicated, almost all the previous investigations on the effect of interruption of the blood supply of the testis have been carried out on this species.

'Chlorbismol' (May and Baker Ltd), a suspension of bismuth oxychloride in 5% dextrose solution, was used as the radiopaque medium throughout this investigation in preference to colloidal bismuth, for while 'Chlorbismol' in 10-20% suspension has particles of a more or less uniform size ($10-12\mu$), the particles of colloidal metallic bismuth in 5 or 10% suspension have the tendency to aggregate to form

a suspension containing particles of unequal size and as much as 28μ in diameter. Hence, while both of these injection media will display only channels which are presumably functional and greater than capillary size, there is a tendency for colloidal bismuth not to fill certain channels due to blockage with the larger-sized particles. 'Chlorbismol', however, will fill the whole vascular bed of an organ, excluding veins and capillaries, quite evenly when injected through its artery, and display subsequently a very clear radiograph. All radiography was done on 'Kodaline' (Kodak nitrate) slow film using a micro-radiography apparatus (Birclay, 1947).

The arterial supply of the rat's testis, as demonstrated by radiography after the injection of 'Chlorbismol' into the thoracic aorta, is shown in Pl 1, fig 1. The testicular artery undergoes convolutions before reaching the testis and first gives off a branch to the fatty body, the epididymis is vascularized either by two separate branches for caput and cauda epididymidis, or, as in Pl 1, fig 1, by a single stem which divides into these two branches. These two vessels always anastomose by a junctional vessel running along the body of the epididymis, and the branch to the cauda epididymidis anastomoses with the vasal artery. The vasal artery may be seen in Pl 1, fig 1, to give off a small tortuous vessel which passes on to the scrotum into the territory of termination of the cremasteric artery. Neither by close examination of the radiographs nor by dissection, however, could there be demonstrated any continuity of the injection mass in the vasal and cremasteric arteries. After giving off its branches to the epididymis the testicular artery appears to be an end-artery, and, after curving around the inferior pole of the testis, convolutes before passing into the testis near its superior pole. Once inside the testis this artery gives off its first branches to the testis parenchyma in its course towards the inferior pole, in this part of its course the artery is lying near the epididymal border of the testis. In no testis out of more than thirty examined was the cremasteric artery filled by retrograde injection after injecting 'Chlorbismol' through the testicular artery.

The appearance of the arterial supply of the dog's testis, as seen by radiograph after the injection of 'Chlorbismol' in six testes at a pressure of approximately 100 mm mercury, is shown in Pl 1, fig 2. On approaching the testis the testicular artery forms a number of convolutions. Before doing so, however, the artery gives off a branch which vascularizes the caput epididymidis. A further branch to the cauda epididymidis is given off from the convoluted part of the testicular artery, which passes along the length of the epididymis to the cauda and here anastomoses with the vasal artery. There are no anastomoses between the testicular artery at the inferior pole of the testis and the vessels on the cauda epididymidis, so that the testicular artery, after giving off its branch to the cauda is an end-artery as far as the testis is concerned. The cremasteric artery is not filled by retrograde injection like the vasal artery, and its communication with the epididymo-vasal anastomosis can, therefore, only be by means of capillaries. This has been found to be the case by Jonanson, Emmel & Pilk (1929), who, however, claimed that there is a well-marked anastomosis between the testicular artery at the inferior pole of the testis and the vasal artery. This has not been found in the testes examined in this investigation.

The testes of the dog and rat are therefore essentially similar in their vascularization in relation to anastomoses. The dog and rat are not the only mammals in which

the cremasteric artery does not appear to enter into functional (i.e. arterial or arteriolar) communication with the testicular and vasal arteries. In fifty separate mammalian species in which the testicular artery has been injected with radiopaque media having particles which do not pass through capillaries, the only two species other than Man in which the cremasteric artery was filled by retrograde injection were members of the Pongidae (the chimpanzee and the gibbon).

Experiments

Material and methods

Fifty-six mature male albino rats, weighing 109–303 g, were used for this investigation. Those rats whose testes were examined histologically are described in Table 1. It can be seen from this table that well-circumscribed operations were performed on the animals. In one group a segment of $\frac{1}{2}$ in. of both testicular artery and vein was removed between ligatures in the abdomen. In another group $\frac{1}{2}$ in. of the abdominal course of the testicular artery only was removed. In a third group a $\frac{1}{4}$ in. segment of the testicular artery, together with the veins of the pampiniform plexus, was removed distal to the point at which the testicular artery gives off its epididymal branches, taking care in the operation to avoid any damage to the branch to the cauda epididymidis. In a fourth group the testicular artery only was ligated with fine nylon thread either at a point on the posterior border of the testis or on the anterior border of the testis just after the artery has passed around the inferior pole, in these situations the artery is running on the deep aspect of the tunica albuginea free from veins, since the testicular veins congregate to a point on the postero-superior aspect of the testis to form the pampiniform plexus. A series of five rats was subjected to interruption of the testicular veins only in the abdomen, and in three rats the artery and vein of the vas were interrupted, carefully avoiding damage to the vas itself. Since Joranson, *et al* (1929) hypothesized, from injection experiments, that increased intratesticular tension, due to the unyielding tunica albuginea, may be an important factor in the production of testicular atrophy following ligation of the testicular vessels, in two further rats the artery and vein were interrupted in the abdomen, while at the same time the tunica albuginea was incised bilaterally.

In all cases the operation on the testicular vessels was performed unilaterally in order to leave the contralateral testis for histological examination as a control. Those animals not described in Table 1, and not examined histologically, were used for investigating the effectiveness of the interruption of the vessels, and any possible post-operative vascular changes, by arteriographic methods.

Results

The results obtained from these experiments are displayed in Table 1. It only remains to summarize and correlate the changes described. On the whole, the cells of the interstitium seem to be more sensitive to vascular changes than the germ cells, in many cases the interstitial cells are degenerated while the tubules contain a few normal germ cells (e.g. rats nos. 723 and 724). The more mature germ cells also seem to be more sensitive to vascular changes than the spermatogonia and Sertoli cells, as described by Schinz & Slotopolsky (1924).

The histological changes following interruption in the abdomen of the testicular artery and vein, or the testicular artery only, occur much less rapidly than after interruption of the artery and vein beyond the anastomosis, or interruption of the artery on the testis. This is reflected in the size of the testis (Table 1, and Pl 1, figs 3, 4) and also by the weight of the organ. Thus in rat no 700 (body weight at death 149 g), in which the artery and vein were interrupted in the abdomen, the weight of the operated testis after 5 weeks was 475 mg (contralateral testis 1065 mg), while in rat no 682 (body weight at death 150 g), in which the artery and vein were interrupted beyond the anastomosis of the testicular with the vasal artery, the weight of the testis was only 302 mg (contralateral testis 1035 mg). Similarly, in rats nos 701 and 681, in which the body weight at death of the former was less than that of the latter (173 as against 221 g) the weight of the operated testis in rat no 701 was 415 mg (contralateral testis 1365 mg), and that in rat no 681, 386 mg (contralateral testis 1434 mg), while in rat no 690 (body weight at death 217 g), in which the artery was ligated on the testis, the weight of the operated testis was 390 mg (contralateral testis 1363 mg). It should be noted in this connexion that the testis of a rat in which the testicular artery and vein are ligated in the abdomen may undergo a preliminary increase in size as the result of vascular congestion (rats nos 721 and 722, Table 1), this does not occur when the vessels are interrupted beyond the anastomosis with the vasal artery (rats nos 702, 703, 723 and 724, Table 1). The histological picture in the operated testes of rats after the same post-operative time interval, shows that, while in rats nos 681, 682 and 690 there was complete degeneration of interstitial tissue and all germ cells in the seminiferous tubules, in rats nos 700 and 701 there was still some interstitial tissue present, although showing fibrosis, and in rat no 700 there were even some tubules containing germ cells up to secondary spermatocytes.

These differences in speed of degeneration are also obvious at later time periods. While, at 7 weeks after operation, the operated testes in rats nos 102 and 104, in which the artery was ligated on the testis, showed complete replacement by fibrous tissue, in the operated testis of rat no 706, in which the artery and vein were ligated in the abdomen 7 weeks previously, there were some normal seminiferous tubules. This last testis is actually the only one in the present investigation which displayed normal seminiferous tubules at any time longer than 4 days after interruption of the testicular artery at any point in its course, and it is important to consider whether these normal tubules have regenerated or persisted unchanged. Five weeks after abdominal interruption of the testicular vessels the testis is markedly degenerated, either the tubules are filled with a mass of eosinophilic substance (rat no 701) or, although containing germ cell elements (rat no 700), these elements appear degenerate and have been desquamated from the walls of the tubules. It is difficult to conceive of such tubules regenerating a normal structure, and it appears more likely that the normal seminiferous tubules in rat no 706 have persisted unchanged or have regenerated after only a partial or minor degree of degeneration. These observations are supported by the fact that in rats nos 700 and 705 the tubules showing the least degenerative changes are those situated along the epididymal border of the testis and that the testicular artery, on becoming intratesticular, passes through the length of the testis towards its inferior pole near the epididymal border of the testis.

Table 1 Summary of the animals used and results obtained from division of the testicular vessels

Rat no	Operation	Post operative time interval	Body weight at death (g)	Size of operated testis (cm)	Size of control testis (cm)	Histology
721	Artery and vein in abdomen	48 hr	237	2 0 × 1 3 × 1 2	2 0 × 1 2 × 1 1	Vascular congestion in interstitium, dilatation of all vessels, particularly of capillaries in interstitium. Most tubules normal except a few at periphery of testis, which show beginning desquamation of cells in tubules, degeneration of cells and appearance of polynuclear giant cells
722		48 hr	208	2 3 × 1 4 × 1 2	1 9 × 1 2 × 1 1	Same as rat no 721, but all tubules show same changes as those at periphery in no 721
704		4 days	111	1 3 × 0 8 × 0 7	1 4 × 0 9 × 0 8	Interstitium shows degeneration. All tubules degenerated. No germ cells beyond secondary spermatocytes. Some tubules only to primary spermatocytes, the cells themselves being degenerate with eosinophilic cytoplasm
705		4 days	143	1 4 × 0 8 × 0 7	1 6 × 0 9 × 0 9	Infiltration of interstitium with macrophages and leucocytes. No germ cells beyond degenerate primary and secondary spermatocytes. Infiltration of interstitium less marked along epididymal border of testis
667		4 weeks	264	1 4 × 0 9 × 0 8	2 0 × 1 1 × 1 1	Fibrosis of interstitium. Those tubules at periphery of testis merely filled with a mass of eosinophilic substance. The central tubules show only spermatogonia and Sertoli cells
700		5 weeks	149	1 4 × 0 9 × 0 8	1 8 × 1 1 × 1 0	Interstitium shows degeneration and fibrosis. Tubules show varying degrees of degeneration, some with spermatogonia and Sertoli cells only, others up to secondary spermatocytes, but all showing desquamation of cells from tubular walls
701		5 weeks	173	1 4 × 0 8 × 0 6	1 9 × 1 1 × 1 1	Fibrosis of interstitium. All tubules filled with a mass of eosinophilic substance. No cellular elements visible at all within the tubules
706		7 weeks	156	1 2 × 0 7 × 0 6	1 6 × 1 0 × 0 9	Majority of testis filled with fibrous tissue, leucocytes and macrophages. A few scattered tubules remain in the centre of the testis, all of which have a thickened membrane propria and show varying degrees of degeneration, some are packed with macrophages and polynuclear giant cells, some with a mass of granular basophilic material, with or without giant cells, and others with eosinophilic substance. Along epididymal border of testis are a few perfectly normal seminiferous tubules showing all stages of spermatogenesis up to and including spermatozoa
707		7 weeks	161	0 7 × 0 4 × 0 4	1 6 × 0 9 × 0 9	The only remaining tubules are a few at centre of testis which are profoundly degenerate containing either eosinophilic or basophilic material with or without polynuclear giant cells and a few at the periphery mainly containing giant cells. Remainder of testis filled with fibrous tissue containing a few macrophages and leucocytes
708		7 weeks	161	0 8 × 0 5 × 0 4	1 9 × 1 1 × 1 1	

676	Artery only in abdomen	1 week	157	$1.4 \times 0.9 \times 0.8$	$1.8 \times 1.0 \times 0.9$	Degeneration of interstitium. Most tubules just filled with eosinophilic substance. A few tubules at periphery of testis show spermatogenous primary spermatocytes and Sertoli cells being desquamated from tubular walls.
671		2 weeks	202	$1.4 \times 0.8 \times 0.8$	$1.9 \times 1.0 \times 1.0$	Interstitial shows degeneration and fibrosis. All tubules show degeneration. Some are just filled with eosinophilic substance, others showing spermatogenous primary spermatocytes, and Sertoli cells being desquamated from tubular walls.
675		4 weeks	211	$1.6 \times 0.8 \times 0.8$	$1.8 \times 1.1 \times 1.1$	Interstitial shows degeneration and fibrosis. All tubules show degeneration. Some are just filled with eosinophilic substance, others showing spermatogenous primary spermatocytes, and Sertoli cells being desquamated from tubular walls.
702	Artery and vein after anastomosis with vaginal artery	48 hr	123	$1.3 \times 0.8 \times 0.7$	$1.4 \times 0.9 \times 0.8$	Interstitial degenerate. Tubules contain degenerate spermatogenous primary and secondary spermatocytes and Sertoli cells.
703		48 hr	129	$1.3 \times 0.8 \times 0.8$	$1.5 \times 0.9 \times 0.9$	
681		5 weeks	221	$1.2 \times 0.8 \times 0.7$	$2.0 \times 1.2 \times 1.1$	Degeneration of interstitium so that tubules in contact with one another. Complete degeneration of all cellular elements in tubules so that tubules merely filled with eosinophilic substance. No adhesions of scrotum to tunica albuginea. As rats nos 681 and 682 but membrana propria breaking down in places so that tubular content running from one tubule to another. No adhesions of scrotum to tunica albuginea.
682		5 weeks	150	$1.1 \times 0.7 \times 0.6$	$2.0 \times 1.0 \times 1.0$	
686		8 weeks	234	$1.0 \times 0.7 \times 0.6$	$2.0 \times 1.1 \times 1.0$	Interstitial degenerate. No germ cells beyond secondary spermatocytes in tubules, in most tubules only primary spermatocytes.
723*	Artery only on testis	48 hr	230	$1.6 \times 1.0 \times 0.9$	$1.7 \times 1.1 \times 1.0$	Interstitial degenerate. All tubules merely filled with eosinophilic substance.
724*		48 hr	268	$1.6 \times 1.0 \times 0.9$	$1.9 \times 1.1 \times 1.0$	
688†		3 weeks	144	$1.2 \times 0.8 \times 0.7$	$1.8 \times 1.1 \times 1.1$	Testis filled with leucocytes and macrophages. Pronounced adhesions all over surface of testis to scrotum and epididymus with ingrowth of capillaries.
450*		4 weeks	295	$1.2 \times 0.8 \times 0.8$	$2.1 \times 1.2 \times 1.2$	Testis just a mass of scar tissue. Pronounced adhesions all over surface of testis.
449*		4 weeks	350	$1.3 \times 0.8 \times 0.7$	$2.2 \times 1.2 \times 1.2$	As rat no 686. Very slight adhesions at surface of testis.
690†	Artery and vein in abdomen with incision of albuginea	5 weeks	217	$1.2 \times 0.9 \times 0.7$	$2.0 \times 1.2 \times 1.1$	Testis just a mass of fibrous tissue. Pronounced adhesions all over surface of testis to scrotum and epididymus.
102†		7 weeks	260	$0.8 \times 0.4 \times 0.4$	$2.0 \times 1.3 \times 1.1$	
104*		7 weeks	276	$0.7 \times 0.5 \times 0.4$	$2.1 \times 1.3 \times 1.2$	
725		4 days	164	$1.2 \times 0.7 \times 0.7$	$2.0 \times 1.2 \times 1.1$	
726		1 week	154	$1.3 \times 0.8 \times 0.6$	$1.8 \times 1.0 \times 1.0$	Interstitial shows pronounced fibrosis. Some tubules filled with eosinophilic substance, others show spermatogenous and Sertoli cells, a few also with primary spermatocytes. Many tubules have escaped through the lesions in the albuginea and show considerable proliferation of fibrous tissue with marked adhesions to scrotum and epididymus.

* On anterior border of testis

† On posterior border of testis

(Pl 1, fig 1) After interruption of the testicular artery and vein in the abdomen, the blood reaching the testis through the vasal artery can only do so by way of the testicular artery. Only those seminiferous tubules in the immediate neighbourhood of the intratesticular part of the artery are therefore likely to receive nourishment if the blood supply to the testis through the vasal artery, by way of the terminal pathway of the testicular artery, is less efficient than the normal supply. There is some evidence that this is the case, since only one testis in eight after a post-operative period of 4 days or more following interruption of the testicular vessels in the abdomen shows any normal seminiferous tubules.

The above findings indicate that the vasal artery in the rat is of some importance in the blood supply of the testis, since the pathological changes following interruption of the testicular artery are less severe when the vasal artery is still able to contribute blood to the testis. The testicular artery, after giving off its epididymal branches, is an end-artery, and its interruption in this situation is followed by rapid degenerative changes. Ligature of the artery on the anterior or posterior border of the testis seems to make little difference in the speed and ultimate outcome of the degenerative changes, as shown particularly by rats nos 102 and 104. This is not surprising in view of the fact that the cremasteric artery is only connected with the vasal artery by way of capillaries (as shown by micro-arteriography after the injection of thorotrast into the thoracic aorta of six rats) across the ligamentum inguinale,* and that no evidence has been found, in the rat, of any vessel larger than capillary size traversing the ligamentum testis*.

There are two predominant histological pictures in the seminiferous tubules following interruption of the testicular artery. In one the nuclei are seen to undergo pyknosis or karyolysis and to disappear eventually leaving the seminiferous tubules filled with a mass of eosinophilic substance, i.e. a process of hyaline degeneration (Pl 1, fig 5). In the other the picture is one of degeneration with replacement fibrosis. These two processes seem to have no definite relation to the operative procedure. Thus replacement fibrosis is the end result of ligature of the artery on the testis in rats nos 102 and 104, but it also occurs in rat no 706 (Pl 2, fig 1) in which the artery and vein were interrupted in the abdomen.

Adhesions of the tunica albuginea to scrotum and epididymis are more common in the cases in which the artery is ligated on the testis. In these cases the testis was necessarily handled in the operative procedure, this being unnecessary in other operations, except in the animals in which the tunica albuginea was incised. Whenever adhesions are found attaching scrotum to tunica albuginea there is an ingrowth of capillaries into the testis, as evidenced by the blueing of the testis following injection of methylene blue solution into the aorta. This was demonstrated in experimental animals in which adhesions occurred after ligation of the artery on the testis, and the ligamentum testis remained unstained in such cases.

The course of the degenerative changes following interruption of the testicular artery only in the abdomen is similar to that in animals in which the testicular artery and vein were interrupted. There is no evidence, therefore, that in the rat

* These terms being used in the sense described by Weber (1927), the ligamentum inguinale being that part of the gubernaculum testis between the cremaster sac and cauda epididymidis, and the ligamentum testis that part between the cauda epididymidis and inferior pole of the testis.

spermatogenesis is more likely to continue after interruption of the artery alone than after interruption of the artery and vein, as claimed for the dog by Wangenstein (1927). This may be explained by the fact that, in the rat, only a proportion of the venous blood from the testis is returned through the testicular veins, a considerable fraction passes directly into the iliac vein by way of a short wide channel. This venous connexion may also explain why, in the five rats in which the testicular veins only were interrupted in the abdomen, there is no diminution in size of the affected testes, and no histological changes up to 4 weeks after operation. Unfortunately, it is difficult to interrupt all venous drainage from the testis in the rat, since it is almost impossible to section all the veins of the pampiniform plexus without damaging also the testicular artery. Section of the vasal vessels alone, up to 4 weeks after operation, produces no changes in the testis. Bilateral incision of the tunica albuginea does not appear to exert any favourable influence on the course of the changes following interruption of the testicular vessels, as shown by rats nos. 725 and 726.

In all cases the tubules of the contralateral testes show histological evidence of perfectly normal spermatogenesis. Examination by arteriography of the vascularization of the testes after the operations used in this investigation displays the complete effectiveness of the operative procedures. Although there is some dilatation of the vasal artery after the interruption of the blood flow through the testicular artery, the degree of this dilatation is variable. Whereas, in some cases the diameter of the vasal artery (as measured on radiographs) is little greater than normal (one-half the diameter of the testicular artery), in other cases the diameter became equal to that of the testicular artery. The degree of dilatation tends to be greater in those cases in which the testicular artery and vein have been interrupted in the abdomen.

THE ANASTOMOSIS OF THE CREMASTERIC AND VASAL ARTERIES WITH THE TESTICULAR ARTERY IN THE HUMAN TESTIS

Practically all the previous work on the effect of ligature of the testicular vessels in experimental animals was performed on dogs, since it was assumed that the blood supply of the testis and epididymis in this animal is similar to that in Man, and that the results obtained from these experiments are therefore applicable to the human testis (see Neuhof & Mencher, 1940). In fact, however, there are marked differences in the manner of anastomosis of the testicular artery with other vessels, as will be apparent from the following account.

Most workers who have made observations on the testicular artery in Man have commented on the cremasteric and vasal arteries (see Harrison & Barclay, 1948, for references), but it was not until the observations of Picqu  & Worms (1909) that the extent and variability of the anastomosis of these vessels with the testicular artery were realized. The investigations of these two workers followed the claim by Mignon (1902) (also, however, made by Bevan, 1903) that, in order to ensure placement of an undescended testis into the scrotum, section of all the vessels in the spermatic cord except those accompanying the vas is often necessary. As a result of their observations, Picqu  & Worms (1909) described the only communication of the vasal artery with the testicular artery as occurring by way of the epididymal branch of the latter, the cremasteric artery was found to anastomose with the other two vessels in all of twenty-four specimens examined, in four cases directly with the testicular

artery, and in the remainder with the loop formed by the epididymo-vasal anastomosis. No indication was, however, given of the functional importance of these anastomoses. An attempt has therefore been made by arteriographic methods to examine the anastomoses of the cremasteric and vasal arteries with the testicular artery, and the possible functional value of these anastomoses.

Material and methods

Twenty-four fresh post-mortem human testes from thirteen individuals were used for the investigation. The ages of the subjects from which these specimens were obtained ranged from 21 to 75 years. The vasal artery was injected in all cases with 'Chlorbismol' in 15 % suspension at a pressure of about 100 mm of mercury, in order that the results obtained may be directly comparable. Radiography was done as in the experiments on the rat testis. Dissection was also used on the injected specimens, and diameters of vessels were measured to the nearest 0.1 mm on the radiographs by taking the average of the values at ten points selected at random along the course of the vessels. As in the experiments on the rat testis, 'Chlorbismol' will only display functional channels of greater than capillary size.

Results

It was soon realized that there are variations in the manner of anastomosis of the three vessels supplying the testis. In every case the type of anastomosis already described in the dog and rat, namely an anastomosis of the vasal artery with the testicular artery by way of the epididymal branch of the latter, is present. But in all of the testes an additional anastomosis is also seen, in which the vasal artery anastomoses with the testicular artery directly at the inferior pole of the testis, without the intermediation of any epididymal branch of the testicular artery. The manner of this anastomosis varies. In fifteen out of the twenty-four testes the anastomosis takes place by means of a large vessel (about 0.5 mm in diameter) which anastomoses directly with one of the major branches of the testicular artery by passing across from the cauda epididymidis to the inferior pole of the testis (Pl 2, fig 2). In the other nine testes the anastomosis is effected by several smaller vessels in this situation which pass into the testis to anastomose with some of the finer intra-testicular branches of the testicular artery (Pl 2, fig 3), or the testicular artery itself, as it is lying on the deep aspect of the tunica albuginea at the inferior pole of the testis (Pl 2, fig 4). In this respect the vascularization of the human testis differs from that of the testes of the dog and rat, which possess no large vascular channels connecting cauda epididymidis with the inferior pole of the testis.

Owing to the two types of anastomosis just described between the vasal and testicular arteries, injection of the former gives rise to retrograde filling of the latter.

However, filling of the cremasteric artery after injection of the vasal occurred in only seventeen out of the twenty-four testes. The anastomoses permitting this retrograde injection of the cremasteric artery, are very complex when present (Pl 2, figs 2, 3). In all cases the cremasteric artery anastomoses directly or indirectly with the epididymo-vasal system of vessels, in six cases this being effected by the cremasteric artery anastomosing primarily with the vessels of the caput epididymidis which then join with the epididymo-vasal system at the cauda

epididymis. This latter anastomosis was effected in four out of the six cases by a slightly tortuous vessel running along the body of the epididymis and seeming to be in direct continuity with the cremasteric artery.

The diameter* of the testicular artery, as measured on the radiographs, varies but little, the smallest diameter observed being 0.7 mm and the largest 1.1 mm (average 0.9 mm). The diameters of the vasal and cremasteric arteries, however, vary within wide limits. Thus the smallest diameter of the vasal and cremasteric arteries is 0.1 mm, the largest diameters being 1.0 and 0.7 mm respectively. The vasal artery may be a mere thread-like vessel (Pl. 2, fig. 4) or a vessel of proportionately large calibre (Pl. 2, fig. 3), while, as has already been noted, the cremasteric artery may fail to enter into functional anastomosis with the other two vessels. It is also interesting to note that the sum of the diameters of the vasal and cremasteric arteries is at least equal to the diameter of the testicular artery in eight out of twenty-four cases in this investigation, while in two cases the vasal artery alone has a diameter equal to that of the testicular artery.

DISCUSSION

There is no unanimity in the literature concerning the effects of division of the testicular vessels in man. Mignon (1902) advocated the section of all blood-vessels in the spermatic cord, except those accompanying the vas, in order to obtain fixation of the testis in the scrotum in the operation of orchiopexy, asserting that in undescended testes the vas is no shorter than normal but the vessels are always very small and form a shortened and inextensible cord. Lucas-Championniere (1902), and Sebileau (1902) claimed, however, that the three cases which Mignon had presented to demonstrate this operation all showed atrophy of the testis. Colles (quoted by Moty, 1902) had previously described the anastomosis between testicular, vasal and cremasteric arteries as being absent in 7% of cases. Bevan (1903) concluded that all vessels in the cord, except those accompanying the vas, could be safely sacrificed in operations for varicocele. Corner & Nitch (1906), in a follow-up of 100 cases of high operation for varicocele in which a 2 in. segment of the pampiniform plexus is removed, observed changes in the consistency of the testis suggesting fibrosis in 90% of cases. No histological observations were, however, made in this investigation. Wells (1948) has pointed out that the testicular artery lies in close relationship to the veins and is likely to be injured if the operation for varicocele is done carelessly; he states that the results of Corner & Nitch (1906) are probably due to the inclusion of the testicular artery in the ligatures, that the operation as commonly described is carried out in such a way as to sacrifice the testicular artery deliberately in the mass division of the pampiniform plexus, and that subsequent atrophy of the testis is no cause for wonder.

Mixer (1924), in describing his operation for undescended testis in children, claims that in 10% of all cases (particularly in the intra-abdominal varieties of undescended testis) the testicular vessels must be sectioned, atrophy of the testis then occurs in 85% of cases. Atrophy may also ensue when the testicular vessels

* In order to diminish distortion of vessel diameter on the radiograph to negligible proportions a tube filament—film distance of 24 in. was chosen. Since the important measurements here are relative to the comparison of the diameters of the vessels the degree of distortion being constant, is not important.

are not divided (12 % of cases), when it is probably due to accidental injury to the vessels at operation

Burdick & Higinbotham (1935) have divided the spermatic cord in 200 cases as an aid to operation on selected types of inguinal hernia. A ligature is placed about the cord at its emergence through the internal ring and a second one as it enters the scrotum, the intervening tissue being excised. It is not specifically stated whether the vasal artery is included in the ligature, and it must only be presumed that the cremasteric artery is left intact. They record clinical atrophy of the testis in only twenty-two out of seventy-nine cases, although a fairly common observation was post-operative swelling of the testis. These results may be criticized on the grounds that no observations were made on the state of spermatogenesis in the operated testes by semen analysis or histological examination by testicular biopsy. Testes recorded as being 'normal in size' need not necessarily display active spermatogenesis, and it may be difficult from clinical examination alone to determine whether the seminiferous tubules are normal. It is significant that all cases in which the cord was divided bilaterally showed normal-sized testes on both sides. Neuhof & Mencher (1940) similarly used complete severance of the spermatic cord (including severance of the vas deferens and its artery) to achieve effective hernioplastic closure in selected cases, the operation is performed in the inguinal canal. They found clinical atrophy of the testis in eight out of nineteen cases. In one case the operated testis was examined histologically, and although 'all stages of spermatogenesis' were evident in some of the seminiferous tubules, they admit of some abnormality in the transformation of spermatids into spermatozoa, and they describe only a few tubules of the epididymis as containing spermatozoa, which had rounded heads and no tails. In absence of further, more definite, observations by semen analysis or testicular biopsy it is therefore difficult to accept unequivocally their conclusion that the human testis is only insignificantly altered following complete division of the cord.

There is, therefore, some lack of agreement as to the effect of section of the testicular vessels. On the one hand, authorities such as Bailey (1936) claim that 'once the spermatic vessels are damaged seriously, atrophy of the body of the testis follows as surely as night follows day', while on the other, some surgeons (Neuhof & Mencher, 1940) claim atrophy in no more than 50 % of cases after complete severance of the cord. On the results of the present investigation several possible explanations of these discrepancies can be made.

Most observers are in agreement that when the testicular vessels supplying a mal-descended testis are damaged atrophy of the testis ensues. But the vessels supplying such a testis are reported as being far from normal, several investigators (e.g. Mignon, 1902) have recorded them as being very small, while Stammmler (1923) has described a hypoplasia of the testicular artery supplying the undescended testis, with intimal proliferation and hyaline degeneration of the walls of its smaller branches, a condition which he believes (probably erroneously) to be primarily responsible for the testicular maldevelopment. Since most operations for undescended testis are carried out on young individuals, another factor to be taken into account is the comparative immaturity and poorer anastomotic connexions of the testicular circulation. This was demonstrated by injection of the testicular artery with 'Chlorbismol' in the testes of two boys, aged 8 and 10 years. In the former only the vasal artery

was filled by retrograde injection through two small anastomotic channels on the body of the epididymis. In the latter neither the vasal nor cremasteric arteries was filled, the epididymis being profusely vascularized by the testicular artery. It can therefore be conceded that any damage to the testicular vessels during orchiopexy may lead to atrophic changes in the majority of cases, particularly since the testis has been in an adverse environment (see Moore, 1926) before operation. The results accruing from section of the testicular artery in such cases consequently should not be applied to herniorrhaphy and varicocele operations in adults, since the testicular circulation in healthy adult men is composed of an efficient arterial anastomotic system in about one-third of all cases. In such cases, in any operation in which only the testicular artery is damaged there is a likelihood that the cremasteric and vasal arteries could take over its function in the presence of an efficient venous drainage. If, however, both the testicular and vasal arteries are sectioned, the only remaining arterial supply to the testis is through the cremasteric artery, since no case has been found in the present investigation in which there is an arterial or arteriolar connexion between the testicular artery and the pudendal vessels. The cremasteric artery in Man is a most variable vessel (functionally probably less efficient than the vasal artery in the rat), as shown, for example, by the complete absence of important anastomotic connexion with the testicular and vasal arteries in about one-third of cases, and it is therefore difficult to envisage this artery alone maintaining an adequate testicular circulation. It also follows that, in the radical operation for varicocele, there will be a greater possibility of survival of the testis if the operation is performed in the inguinal canal than if it is carried out below the external ring, in the case of inadvertent damage to arteries (always providing that the vas and its vessels are avoided) the danger of interruption of the testicular artery would be greater below the external ring. In this position the testicular veins have a more complicated arrangement than in the inguinal canal, the nearer the testis the more voluminous the pampiniform plexus, and since the testicular artery is always ensheathed by the veins of pampiniform plexus there is greater difficulty in isolating this vessel near the testis. The venous drainage, although of less importance due to the greater profusion of anastomotic connexions, must also be considered. Javert & Clark (1944) maintain that the optimum point of ligation of the testicular veins in operations for varicocele is at the internal abdominal ring, which, while eliminating the static column of blood does not impair the collateral circulation through the cremasteric and vasal veins. The venous circulation through alternative channels to the testicular veins has already been noted as of no small importance in the rat.

Finally, it is not valid to apply unconditionally the results of animal experiments to the problems resulting from ligation of the testicular vessels in Man. Animal experiments can only give some indication of the pathological processes involved. It has been seen that the vasal artery which, in the rat, is only one-half the diameter of the testicular artery, can play a small part in the delay (and occasionally partial circumvention) of degenerative processes. In Man, however, the vasal artery is much larger, proportionately, than its counterpart in the rat, has more efficient anastomotic connexions with the testicular artery, and has the assistance of the cremasteric artery in some cases in the nourishment of the testis. The results of division of the testicular artery must therefore be very different in Man and other mammals. It

has also been noted (Harrison & Barclay, 1948) that the testicular artery in Man (at least in the European) differs from that in the majority of mammals by being almost straight in its course to the testis

The results of incision of the albuginea when the testicular vessels are interrupted do not seem to be encouraging in the rat. The claim by Baker & Evoy (1942) that 'decompression' of the human testis, by incising the albuginea in one case of painless enlargement following herniorrhaphy, produced 'no discernible abnormality of the involved testicle', in contrast to a similar, untreated case, must therefore await histological confirmation.

Once the testicular artery or its branches have passed into the testis, on the deep aspect of the tunica albuginea, such vessels are functionally end-arteries after the anastomosis at the inferior pole of the testis. Consequently, the conclusion (Harrison & Barclay, 1948) that great care must be exercised to avoid ligaturing the testicular artery in its superficial position under the tunica albuginea in the surgical treatment of cryptorchids, still obtains.

SUMMARY

1 The degenerative processes resulting from interruption of the testicular artery at various points in relation to its anastomotic connexions have been examined in the rat. The results indicate that the vasal artery in the rat is of some importance for the blood supply of the testis, since the degenerative changes following interruption of the testicular artery are less severe when this artery is still able to contribute blood to the testis.

2 The vascularization of the testis in the dog and rat differs from that in Man in that a direct anastomosis of the testicular artery with the vasal and cremasteric arteries is absent. Unconditional application of the results of division of the testicular vessels in experimental animals to Man is therefore not valid.

3 Examination by arteriographic methods of the vascularization of the human testis demonstrates that the sum of the diameters of the cremasteric and vasal arteries is at least equal to the diameter of the testicular artery in one-third of all cases examined. In approximately one-third of cases the cremasteric artery does not enter into functional anastomotic connexion with the testicular and vasal arteries.

4 The importance of these observations for the results of division of the testicular artery in Man is discussed, and several possible explanations of the discrepancies in the literature concerning the effects of ligature of the testicular artery in Man are proposed.

I am indebted to the late Dr A. E. Barclay, and Dr G. S. Dawes for permitting the use of the micro-radiography apparatus at the Nuffield Institute for Medical Research. I also wish to thank Mr L. G. Cooper for his technical assistance. May and Baker Ltd., kindly supplied all the 'Chlorbismol' used.

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EXPLANATION OF PLATES

PLATE 1

- Fig 1 The arterial supply of the rat testis as visualized by radiography after the injection of 'Chlorbismol' into the thoracic aorta. Natural size. *ta*, testicular artery, *va*, vasal artery, *ca*, cremasteric artery. The testicular artery convolutes before reaching the testis, and gives off branches to epididymis and fatty body. The branch to cauda epididymidis anastomoses with the vasal artery and this anastomosis sends off a small branch on to the scrotum into the territory of terminal distribution of the cremasteric artery, not, however, forming a functional anastomosis with it.
- Fig 2 The arterial supply of the dog's testis as visualized by radiography after the injection of 'Chlorbismol' through the testicular artery. Natural size. *ta*, testicular artery, *va*, vasal artery. The testicular artery convolutes before reaching the testis, and its branch to the cauda epididymidis anastomoses with the vasal artery. There are no vessels passing from cauda epididymidis to inferior pole of testis.
- Fig 3 The appearance of operated (on left) and unoperated (on right) testes 4 weeks after interruption of the testicular artery and vein in the abdomen ($\times \frac{4}{3}$).
- Fig 4 The appearance of operated (on left) and unoperated (on right) testes 4 weeks after ligation of the testicular artery on the posterior border of the testis. Note that the epididymis on the operated side is normal in size ($\times \frac{4}{3}$).
- Fig 5 Photomicrograph of the seminiferous tubules in the testis of a rat (682) in which the testicular artery and vein were interrupted after the anastomosis with the vasal artery 5 weeks previously. The interstitial tissue is degenerate, and there is pronounced hyaline degeneration of the contents of the tubules, no germ cell elements persisting ($\times 112$).

PLATE 2

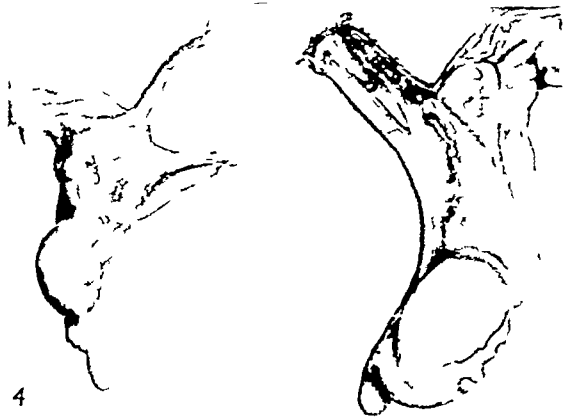
- Fig 1 Photomicrograph of the testis of a rat (706) in which the testicular artery and vein were interrupted in the abdomen 7 weeks previously. The figure shows an area in that part of the testis in which there is considerable fibrosis with some infiltration of leucocytes and macrophages. Only a few degenerate tubules remain, in one of the tubules a polynuclear giant cell may be seen ($\times 86$).
- Fig 2 Radiograph of the left testis and spermatic cord from a man aged 71 after the injection of 'Chlorbismol' into the vasal artery (*va*). This artery enters into anastomosis with epididymal branches of the testicular artery (*ta*) and also directly with the testicular artery towards the inferior pole of the testis. The cremasteric artery (*ca*) is also injected. Natural size.
- Fig 3 Radiograph of the right testis and spermatic cord from a man aged 58 after the injection of 'Chlorbismol' into the vasal artery (*va*). The cremasteric artery (*ca*) is clearly seen. The anastomosis formed by the vasal artery and epididymal branches of the testicular artery (*ta*) sends off several small vessels into the testis to anastomose with the finer intratesticular branches of the testicular artery. The arrows point to the anastomoses made by the cremasteric artery with the other two vessels. A branch is given off from the cremasteric artery which, at the upper arrow, is seen to send off a side branch to the epididymo vasal anastomosis, and then pass on to anastomose with the vasal and testicular arteries directly. The terminal branches of the cremasteric artery pass to the cauda epididymidis and here (at the lower arrow) are seen to join with the epididymo vasal anastomosis. Natural size.
- Fig 4 Radiograph of the left testis and spermatic cord from a man aged 75 after the injection of 'Chlorbismol' into the vasal artery (*va*) which is seen here to have divided into two vessels of smaller diameter. The cremasteric artery (*ca*) is a very small vessel. The epididymo vasal anastomosis forms a complex network of small vessels at the cauda epididymidis, from which a few fine vessels pass across to the inferior pole of the testis to anastomose directly with one of the branches of the testicular artery (*ta*). Natural size.



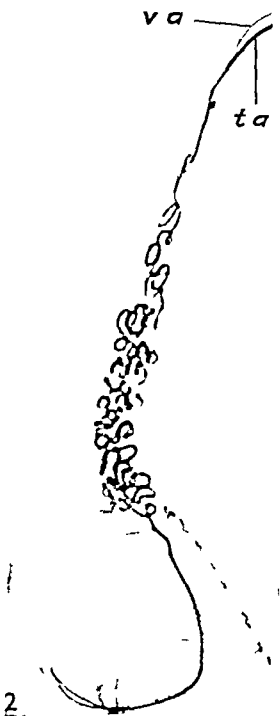
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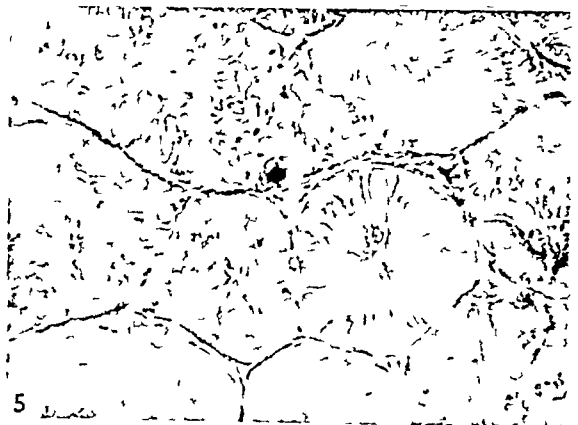
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The value of any anatomical atlas must remain largely a matter of personal prejudice, for probably no two opinions will agree as to optimum scope of content. The present atlas appears uneven in this particular, for some structures of the chosen region receive a treatment more elaborate than that bestowed upon others of equal importance. Thus four figures (Figs 96-99) are devoted to enlarged reproductions of the auditory ossicles (without their associated ligamentous or vascular apparatus), yet the tympanum itself is accorded a less liberal attention. The thyroid gland receives no particularly detailed treatment, whilst the parathyroids are altogether omitted, and the utility of four cerebellar figures in a work of this nature seems questionable.

Anatomical blemishes appear to be few. We note, however, that in Fig 6 the ala nasi is not innervated by the maxillary nerve, while the auriculo-temporal nerve supplies overmuch of the skin of the pinna. The jugular lymph trunks and the submental lymph glands are nowhere figured. An obvious socia parotidis is wanting in Fig 18. The superficial Sylvian vein is depicted (Fig 31), but the deep Sylvian and the basal veins are ignored elsewhere. In Figs 24, 25 and 65 the sinus of Morgagni is not labelled. The cervical vertebrae (Figs 83-85) are practically devoid of metapophyseal tubercles and their anterior ligamentous areas are too feebly pronounced. The atlas vertebra (Fig 68) is given a 'lateral process', apparently a new term for the posterior bar of the transverse process. The upper limit of origin of *M. temporalis* in Fig 91 is not coincident with the inferior temporal line. The picture of the basis crani interna (Fig 94) shows the petrous to be devoid of its customary vascular imprints from the superior and inferior petrosal sinuses and the sphenoidal lingula is insufficiently emphatic. The external auditory meatus (particularly the tympanic plate) is poorly represented in Figs 90, 91. These illustrations, together with Figs 92, 94, demonstrate the general unsuitability of monochrome 'wash' as a medium for depicting osseous structures or tissue. It is unfortunate that in Fig 104 the several elements bounding the ostium maxillare remain unlabelled and that the dissection of the temporal bone was not carried to a deeper and more informative level.

The standard of artistic execution is high and reflects a commendable fidelity to the original preparations. The restriction, however, of bright colours to neural and vascular structures only is perhaps not altogether happy. For (in the horizontal and frontal sections particularly) these stand out too vividly against a general monochromatic background and the eye is deflected by minor branches and twigs from more important structures. Had the muscular, glandular and other masses been appropriately tinted this defect would have been obviated.

The quality of the paper, the printing and the general production are superb, and there is an exhaustive index. Every accurate reproduction of a good dissection or section is of value, and this atlas should therefore prove serviceable to the medical student. We doubt, however, whether the ophthalmologist, the otologist and the laryngologist will find it sufficiently comprehensive for professional reference.

A. J. L. CANN

BONES, MUSCLES AND VITAMIN C

III REPAIR OF THE EFFECTS OF TOTAL DEPRIVATION OF
VITAMIN C AT THE PROXIMAL ENDS OF THE TIBIA AND
FIBULA IN GUINEA-PIGS

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The changes to be described occurred at the proximal ends of tibial and fibular diaphyses of guinea-pigs which were first subjected to a period of total deprivation of vitamin C, and then supplied with the vitamin. The methods and material used are described in the first paper of this series (Murray & Kodicek, 1949*a*, p 158), and the experiments are summarized in Table 1 of that paper (p 160)

The changes occurred in two phases—a destructive phase followed by one of repair

THE DESTRUCTIVE PHASE

This phase occurred only in animals which suffered a period of total deprivation of vitamin C

It is well known that the pathological changes in scurvy include the cessation of ossification at the growth cartilage and elsewhere, the appearance of microfractures at the ends of the diaphyses of long bones, and even the detachment of epiphyses. Since it was our object to keep the animals alive until well after the time when the vitamin was put back into the diet, we have little histological material which would enable us to confirm the classical descriptions of these effects of total deficiency, we have, however, radiographs. Those, made immediately or shortly after the end of the period of total deficiency, show unmistakable evidence of the changes mentioned. In normal guinea-pigs, radiographs show the proximal end of the tibial diaphysis widening gradually to the growth cartilage, like a trumpet to its mouth, and the epiphysis fitting neatly over the diaphysis without overlapping it (Text-fig 1A, and first paper, Pl 1). On the other hand, in many guinea-pigs of groups 3, 4, 5 and 6 of Exps 5 and 6, the first radiographs, made after 9 days of total deprivation of vitamin C followed by seven (Exp 6) or eight (Exp 5) days on 0.5 mg ascorbic acid daily, showed the proximal epiphysis of the tibia overlapping the diaphysis medially, or both medially and laterally, while the diaphysis itself more or less completely lacked its normal proximal expansion (Text-fig 1B, Pl 1, figs 1A, 2A, 3A, Pl 2, fig 12A). The change was usually greater on the medial than on the lateral side. It was observed in nineteen of the fifty-four legs. Similar changes were seen in Exps 7 and 8.

* This work was done while the first author was at the Department of Biology, St Bartholomew's Hospital Medical College.

In addition, the epiphysis, which in normal animals sits squarely on the top of the diaphysis in a plane at right angles to the long axis of the shaft, was in many cases tilted downward, nearly always on the medial side (Pl 1, fig 1, etc) Since it retains its normal relation, through its articular surfaces, ligaments, etc, with the femur, its sloping attachment to its own diaphysis causes the animal to be bow-legged, compare Pl 1, fig 4, a pair of normal limbs, with fig 5 Sloping epiphyses were seen in nineteen of the fifty-four legs, and nearly always in the same legs which also showed epiphyseal overlap

The loss of the diaphyseal expansion, and the medial slope of the epiphysis, were accompanied by the appearance of an irregular shadow usually extending across the diaphysis immediately below the growth cartilage, and by the presence, in the angles between the end of the diaphysis and the overhanging edge of the epiphysis, of small spicule-like shadows strongly suggestive of outwardly fractured bits of the proximal end of the diaphysis

From this picture, and from the classical descriptions of scurvy in guinea-pigs, it was clear that the gently widening proximal end of the tibial diaphysis had been destroyed, that it was now represented by debris which gave the X-ray shadows just mentioned, and that the region of the diaphyseal wall now in contact with the epiphysis was not its normal proximal end (Text-fig 1A), but a narrower region which would normally be situated more distally Hence the epiphyseal overlap (Text-fig 1B) The slope of the epiphysis obviously reflects more extensive destruction of the medial wall of the tibia than of the lateral wall, the lateral wall is probably to some extent protected by the fibula whose proximal epiphysis is in guinea-pigs fused with that of the tibia

Radiographs taken later in the experiment may show the condition aggravated, sometimes in an extreme degree, thus, the diaphysis might fracture almost right across just below the growth cartilage (Pl 3, fig 14B) Such severe changes were rare, but there was often an increase in the medial downward slope of the epiphysis By far the commonest and most interesting changes, however, were constructive, and led to the formation of the structures described below

THE PHASE OF REPAIR

The reparative changes occurred only in those animals to which, after the period of total deprivation, a supply of vitamin C was again allowed The amount of the vitamin given varied in the different experiments (Exps 5-8, Table 1), it is of interest that the repair occurred when the amount of ascorbic acid provided was 0.5 mg daily, apparently as well as when it was 10 mg, although the smaller amount did not usually allow normal formation and consolidation of fracture calluses, subperiosteal thickenings, too, remained unconsolidated

In radiographs taken at various times after the restoration of the vitamin to the diet, the proximal end of the tibial diaphysis showed thickenings of its wall which sometimes appeared to be purely periosteal in origin (Pl 1, figs 1, 2) but sometimes also endosteal The new bone thus formed tended to fill in the gap between the overhanging epiphysis and the diaphysis below it, and often appeared to form a callus-like mass within the diaphyseal cylinder just below the growth cartilage The general effect was to restore the tibia to something like its normal form, but the epiphyseal

slope and resulting bow-leggedness were not corrected during the course of our experiments

We studied these changes almost exclusively in the tibia, giving but little attention to the fibula, in which changes of a generally similar character appear to have occurred, because it is much easier to study and interpret the alterations in the larger bone. The radiographs gave no indication that similar changes, either of a destructive or of a reparative character, occurred at the distal ends of these bones, and we cut no sections of this region

Sections were prepared of fifteen representative specimens. They were mostly frontal serial sections through the proximal ends of the tibia and fibula, but some included the distal end of the femur and some were cut in other planes. The material was all taken from animals which, after a period of total deficiency of vitamin C, were again supplied with the vitamin. Table 1, in conjunction with Table 1 of the first paper (p. 160) (Murray & Kodicek, 1949*a*) will indicate sufficiently the history of these animals

Table 1

Exp	Group	No of animals	No of days from day 1 to death	No of days from restoration of vitamin C to death
5	3	3	63, 113, 121	45, 95, 103
	4	2	45, 62	27, 44
	6	1	61	43
6	4	2	30, 85	14, 69
7	1	2	62, 64	52, 54
	2	3	107 (3 animals)	97 (3 animals)
8	2	2	66 (2 animals)	50 (2 animals)

It will be simplest to begin the description of the structural changes revealed in the sections by describing specimen 'VA 40' which died earliest after restoration of the vitamin. This animal died 13 days after transference from a diet without vitamin C to one containing 0.5 mg. ascorbic acid daily. It is of interest because it still shows, in some degree, the histological counterpart of the destructive changes which the radiographs indicate as occurring during the total deprivation of the vitamin, not yet obscured by the effects of the added ascorbic acid, and because it also shows earlier stages in the processes of repair than are found in animals which survived longer. The sections (Pl. 1, figs 7, 8, Pl. 2, fig. 9) are frontal and show the tibial wall, both laterally and medially, not continuing as it normally does (Pl. 1, fig. 6, Text-fig. 1A) to the level of the growth cartilage, but ending below it in a fibrous connective tissue ('Fasergerüst') which locally replaces the normal haematopoietic marrow. In this tissue, scattered near the upper end of the tibial shaft, are small pieces of bone. Some of these seem to be degenerating, we believe them to be detached fragments of the diaphyseal wall, broken off by microfractures during the total lack of the vitamin, or soon afterwards. Pl. 1, fig. 8 and Pl. 2, fig. 9 show similar fragments evidently just broken off, one in the tibia and one in the fibula. Also lying in the same connective tissue are islands of development of new bone of the granular type mentioned in the second paper (Murray & Kodicek, 1949*b*) in connexion with the mid-diaphyseal thickenings and their development.

Occupying the extreme proximal end of the tibial marrow cavity are lightly built bony trabeculae, many of them enclosing vestiges of the matrix of the growth

cartilage, at which they have evidently been formed by endochondral ossification. This bone must have been formed since ossification started again after restoration of ascorbic acid to the diet. The endochondral trabeculae here formed are being strengthened by the deposition of endosteal bone on their surfaces and by the formation of new endosteal trabeculae.

The tibial shaft showed (Pl 1, fig 7), both laterally and medially, a widened periosteal cambium with new bone forming in it just as in the development of a mid-diaphyseal thickening. This new bone was not revealed by the radiographs and so was presumably incompletely calcified.

The widened cambial tissue ended proximally at a mass of cartilage (Pl 1, fig 7, *peri cart*) which, like the new subperiosteal bone, is not normally present. This cartilage, which is seen both laterally and medially, lay between the fibrous layer of the periosteum and the bony shaft and callus, at the proximal end of the tibial diaphysis. Its tissue passed by a gradual transition into that of the periosteum from which it was evidently formed. The internal cells of the cartilage were hypertrophied and the matrix was basophil, while those nearer the surface were small and lay in an almost colourless matrix (haematoxylin and eosin). Radiographs showed that the matrix was calcified. In places the cartilage had a tenuous connexion with the epiphyseal cartilage which thus had the appearance of being turned downwards at its rim, around the end of the diaphysis, much as a match-head grips the stick. It is not being resorbed and no endochondral ossification is occurring in it.

The condition of the fibula, in the region of the growth cartilage, in general resembles that of the tibia, in particular, it shows a very beautiful microfracture of the bony shaft (Pl 2, fig 9).

We thus see in this specimen (a) a widened subperiosteal cambium with new bone developing in it, (b) a bony callus-like tissue forming endosteally in the splintered proximal end of the tibial shaft, (c) the formation of trabecular bone from the growth cartilage, and (d) a differentiation of cartilage from the periosteum in the same region.

When we turn to the remaining specimens of which sections were cut (Table 1), all of which survived for longer periods than that just described, we find that their radiographs showed a great variety in the amount of reparative hard tissue formed at the proximal ends of the tibiae. Thus, in the specimen illustrated in Pl 2, fig 10B the medial wall of the tibia showed a thickening which increased towards the proximal end, stopping rather abruptly a little below the growth cartilage, but showed no other change except the medial slope of the epiphysis. It is notable that the thickening ceases proximally at a position corresponding with that of the top of the diaphyseal wall in the first photograph (fig 10A) (taken just before the end of the total deficiency), as indicated by the distance from the point at which the medial wall of the diaphysis curved laterally. Pl 1, fig 1A, B, show somewhat greater changes, with subperiosteal new bone running up to the epiphysis on both sides, a medial slope of the epiphysis, and a suggestion (confirmed by sections) of endosteally formed callus. The bones shown in Pl 2, fig 12A, B, Pl 3, fig. 13A, B, and fig 14A, B show generally similar but greater changes: thickening of the proximal tibial wall in relation to epiphysial slope, overlap caused by the destruction of the proximal ends of the tibial diaphyses, and a suggestion of internal callus formation. In the specimen shown in Pl 3, fig 14A, B the extreme degree of over-

lap of the epiphysis on the medial side, caused by breakdown of the diaphysis, led to a fracture which sections show to have been nearly completely across the bone

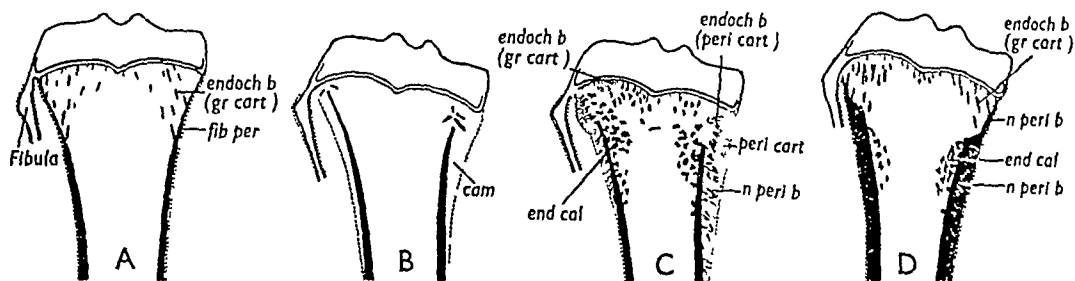
Sections show that the changes in these and other specimens must be referred to the four processes discerned at work in the specimen which died earliest, and which is described above. We shall discuss these processes *separatim*

Formation of the subperiosteal thickening

In the specimen which died at 13 days ('VA40') after transference to a diet containing vitamin C, the subperiosteal thickening developed in a widened cambium. In other specimens, all of which lived longer after restoration of the vitamin, comparison of the earlier radiographs with the structure revealed by the sections, showed that a periosteal thickening had formed in the same way. In these sections it was possible to distinguish the bone formed in the subperiosteal thickening, and that of the endosteal callus developed in the marrow cavity, from the old bone of the original diaphyseal shaft, by the more regular orientation of the cells and fibrous structure of the old bone and by its staining properties, in which it differed slightly from the more recently formed bone. In spite of resorptive changes the old diaphyseal wall could usually be traced far enough towards the growth cartilage for it to be obvious by extrapolation that it pointed, not to the edges of the growth cartilage, but to places within the perimeter of the latter (Pl 2, fig 12D, E). The sections thus confirmed what the earlier radiographs showed, that at an earlier time the epiphysis had overlapped the proximal ends of the diaphysis. Now, the periosteum normally ends proximally where its fibres pass into the epiphyseal cartilage, just proximal to the growth cartilage, and into the joint capsule, from this attachment it normally descends along the diaphysis, lying close against it. But evidently when the diaphysis has lost its proximal end, the periosteum must tend to be loose over the angle between the diaphysis and the overlapping epiphysis. Now, after a fracture of the shaft of a normal guinea-pig's fibula the damaged periosteum re-establishes itself around a cellular mass which develops in the oedematous and haemorrhagic area between and around the broken ends. Because of the local haemorrhage and oedema, the newly re-established periosteum is displaced outwards from the bone on which, therefore, the callus forms a thickening. Similarly, the oedema developing in connexion with the damaged proximal end of the tibial diaphysis must tend to swell the periosteum locally outwards. Thus the anatomical changes brought about in the destructive phase, and the resulting oedema, combined to produce a widened subperiosteal space around the proximal end of the diaphysis, this space filled with osteogenic cambial tissue, and the proximal thickening resulted. It was clear from the relation of the periosteum to the angle beneath the overhanging epiphysis and to the diaphysis that the new bone thus formed tended to re-establish the normal widening of the proximal end of the tibial shaft (see Text-fig 1B).

It would be supposed that the new bone would have functional value in supporting the overlapping epiphysis and (in terms of theories of functional determination of bone structure) might be regarded as brought into being by mechanical stimulation of a functional nature. While it is obvious that the new bone might have the functional value suggested, in that its trabeculae could make direct contact with the epiphysis and transmit strain from it, three considerations forbid us attributing its formation

to this stimulation. First, sometimes such contact seemed to be lacking, any strains carried from the epiphysis to the new bone being transmitted to it through connective tissue which seemed quite unfitted to such a function. Secondly, the trabeculae tended to be orientated radially and downward, from the old bone to the fibrous layer, and this is a direction roughly perpendicular to that expected if they were weight-carrying piers. Thirdly, in the early stages of its development the new bone could hardly discharge such a function because it was little if at all calcified, a bony structure can only develop in response to mechanico-functional stimulation if either the strains develop *pari passu* with the bone, or if, as in endochondral ossification, the bone takes over the function of some pre-existing structure which has hitherto borne the strain.



Text fig 1 Diagram of the changes at the proximal end of the tibial diaphysis in an animal supplied with vitamin C after a period of total deprivation. A Proximal end of normal tibia and fibula, compare Pl 1, fig 6. B After a period of total deprivation of vitamin C, microfractures at the proximal end of the tibial diaphysis reduce its length, especially laterally, the epiphysis is made to slope downward laterally, and the cambial zone (*cam*) of the periosteum is widened. C During repair. New subperiosteal bone (*n peri b*) forms in the widened cambium. Periosteal cartilage (*peri cart*) and endosteal callus (*end cal*) are seen. Endochondral bone (*endoch b*) is forming by replacement of the periosteal cartilage and from the growth cartilage. Compare Pl 1, fig 7. D The new subperiosteal bone has been made compact, periosteal cartilage has been resorbed, and the endosteal callus partly so, normal activity of growth cartilage. The most recently formed part of the diaphyseal wall (at the top) is of the normal thickness. Compare Pl 4, fig 16C. Fibrous layer of periosteum (*fib per*) dotted.

The development of subperiosteal thickenings on the fibula might be somewhat differently determined, Pl 2, fig 9 shows the development of new bone in an angle between periosteum, epiphysis, and old bone, created by a bend in the fibula at the level of the growth cartilage.

The periosteal cartilage

The cartilage was always formed at the top of the diaphysis and below the growth cartilage, internal to the periosteum and external to the proximal end of the diaphysis or to the callus in which the diaphysis ends (Text-fig 1C). It was formed by local chondrification of the periosteum, into which it passed peripherally by a gradual transition. It was usually continuous with the cartilage of the epiphysis, and often looked as if formed by the epiphyseal cartilage being bent round, or having flowed round, the end of the shortened diaphysis, this was suggested, for example, in Pl 2, fig 9. We do not think, however, that this appearance can indicate an actual histogenetic process because of the clear evidence of periosteal origin of the cartilage and because of the difficulty in supposing the differentiated epiphyseal cartilage to possess such great mobility.

The cartilage appeared in about half the tibiae studied, and especially in those showing the greatest disturbance of the normal structure. As in the specimen which died earliest ('VA 40'), the cells in the middle of the cartilage hypertrophied and the matrix here became basophil, in specimens kept for longer periods the hypertrophy spread to the surface facing the bone and here the cell chambers were opened and endochondral ossification occurred (Pl 3, fig 15B). Peripherally, where the cartilage passed over into the periosteum, the cells remained small and the matrix eosinophil, or at least the enlargement and basophilia did not occur till later. The bone formed by replacement of this cartilage was added to the endosteally formed callus in which the old diaphyseal bone could often be seen to end (Text-fig 1C). Its extent could be estimated from the vestiges of cartilage matrix embedded in the trabeculae of bone, and in one or two cases the presence of these revealed that a part of the callus had developed in a cartilage which had otherwise been totally destroyed by the time of fixation.

The cartilage must be compared with that formed in most fracture calluses, also from the periosteum or its derivative cells. There is strong evidence that connective tissue structures which do not normally form cartilage can be caused to do so by rather ill-defined mechanical conditions in which they are subjected to pressure. This has been shown for embryonic material *in vitro* by Glücksmann (1939), and, in tendons subjected to lateral pressure, by Ploetz (1937-8), while Krompecher (1937) found that cartilage formation at experimental fractures was prevented when the broken ends were pulled apart. In the present experiments it has repeatedly been seen, in both dietetically normal and partially deficient animals, that where the tibia and fibula come into contact and presumably press on each other, the periosteum of both chondrifies. It is thus possible, and in accord with other evidence, that the chondrification of the periosteum at the proximal end of the tibial diaphysis may be a reaction to the pressure of the internal oedema upon it. It is perhaps significant that it formed at the extreme proximal end of the shaft, where microfractures and resorptive changes deprived the periosteum of protection by an intact diaphyseal wall beneath it from the action of pressures acting from within the bone. Possibly it may have been a reaction to pressure downwards from the epiphysis, but it had not this appearance. The consequence of its formation and later replacement by bone was to add to the bony callus which, with the new subperiosteal bone, the endosteal callus, and the bone formed by renewed activity of the growth cartilage, completed the restoration of the connexion between the epiphysis and the diaphyseal shaft. Further, since the cartilage was formed from the periosteum, its replacement by bone continued the widening of the new proximal end of the shaft, and therefore helped to restore the normal form of the tibia.

Endosteal callus

This was trabecular bone developed endosteally, especially about the shattered upper end of the tibial diaphysis. It formed a large part of the callus developed in this region, the remainder being formed partly by replacement of the periosteal cartilage and partly by endochondral ossification at the growth cartilage. Often, especially when it is large, the callus develops in the fibrous connective tissue by which the haematopoietic marrow is partly replaced, but sometimes it forms in

unaltered haematopoietic tissue The quantity of endosteal callus formed differed greatly in different specimens, presumably depending on the severity and detailed nature of the injury It might extend right across the marrow beneath the growth cartilage, or for a considerable distance along the inner aspect of the shaft wall, limiting the extent of the marrow cavity (Pl 2, fig 12C, Pl 3, figs 13C, 15A, and compare Pl 4, fig 18 with fig 17, normal) We can offer no explanation of its occasional extent so far from the seat of injury

When the old bone of the diaphysis had been so far destroyed as not to constitute a barrier between them, the endosteal callus and the subperiosteal thickening united, but the approximate position of the boundary between them could usually be detected by the more regular radiating arrangement of the subperiosteal trabeculae, those of the endosteal bone being usually much less regularly orientated

In animals which lived for long periods after the restoration of vitamin C to the diet, it was sometimes found that the callus lay at some distance below the proximal end of the diaphysis, in such cases the callus must have formed at the proximal end soon after the restoration of the vitamin and then, comparatively normal growth being resumed, a new length of shaft was formed above it by the growth cartilage and periosteum (Text-fig 1D, Pl 4, fig 16C)

The activity of the growth cartilage

The growth cartilage, which must be supposed to have been inactive during the period of total deprivation of the vitamin, in the sections showed resumed activity and endochondral bone was seen in formation This activity, because of the diameter of the cartilage, must evidently have led to the development of a new proximal end of the diaphyseal shaft, and this must evidently have had the diameter of the growth cartilage, i.e. a diameter greater than that of what had, during the destructive phase, become the proximal end of the shaft The formation of a fairly smooth junction between the wide new proximal part of the shaft, and the narrower old part now just distal to it (Text-fig 1B, C, D), was brought about partly by the new subperiosteal bone, partly by the new bone formed endochondrally at the periosteal cartilage, partly by the endosteal callus, and partly of course by the modelling process which normally brings about the transformation of the wider proximal parts of the shaft into the narrower more distal part

At first, the bone formed at the growth cartilage contributed to the internal callus, but as growth proceeded the cartilage was carried away from the callus, and a gap appeared between the recently formed endochondral bone and the internal callus This gap had presumably earlier contained endochondral bone which had been resorbed, as occurs in normal growth (internal spongy bone being present only at the ends of most long bones)

A similar effect could be observed in connexion with the subperiosteal thickening, thus, in Pl 2, fig 10C the most proximal part of the lateral wall of the shaft was of about normal thickness, but at a short distance from the growth cartilage the wall abruptly thickened This thickening was caused by subperiosteal bone developed as usual beneath an epiphysis overhanging a shortened and therefore abnormally narrow diaphysis, but, as soon as the growth cartilage became active and formed

a diaphyseal end of normal diameter, no widened subperiosteal space existed and as the new wall was formed it was of normal thickness

SUMMARY

1 When guinea-pigs were subjected to total deprivation of vitamin C they showed the classic changes and, since the proximal end of the tibial diaphysis was destroyed by repeated microfractures, the epiphyses came to overlap, both laterally and medially, the narrow zone of the tibial shaft now in contact with it. The damage to the diaphysis was usually greater medially than laterally, causing the epiphysis to slope downwards and medially, making the animals bow-legged.

2 When animals, in which these changes had occurred during a period of total deficiency, were again given the vitamin, reparative changes restored the tibia to a form approaching the normal. These changes were (a) the formation of a subperiosteal thickening in the widened periosteal cambium which filled the angle between the overhanging epiphysis above, the fibrous layer of the periosteum, and the old diaphyseal wall, (b) the formation, around the proximal end of the diaphysis, of cartilage derived from the periosteum, and its later replacement by endochondral bone which was added to (c) a trabecular bony callus developed endosteally, and (d) trabecular bone formed endochondrally at the growth cartilage. All these changes occurred in some cases, but not in every case, and there was great variation in detail.

We wish to thank Mr P. O. Mumby, F.R.M.S. for taking the photomicrographs, and to Mr S. G. Impey for his efficient care of the experimental animals.

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EXPLANATION OF PLATES

PLATE 1

- Fig. 1 From a partially vitamin C-deficient guinea-pig of Exp. 5, group 4. A. 27th day of experimental diets, 9th day on 0.5 mg. ascorbic acid daily, day of operation (fracture just visible in original). On the medial side, the proximal epiphysis overlaps the diaphysis, which lacks its normal curvature. B. 47th day of experimental diets, 20 days after operation, 29th day on 0.5 mg. ascorbic acid daily, day of death. Thickening of proximal end of tibial diaphysis, medial slope of epiphysis, no radiologically recognizable callus at fracture.
- Fig. 2 From a partially vitamin C deficient guinea pig of Exp. 5, group 6. No operation. A. 27th day of experimental diets, 9th day on 0.5 mg. ascorbic acid daily. The proximal end of the tibial diaphysis has lost the normal outward flare of the walls, and a heavy shadow lies below the epiphysis. B. 44th day of experimental diets, 26th day on 0.5 mg. ascorbic acid daily. Subperiosteal thickening of both tibial and fibular diaphyses, except distally, medial slope of tibial epiphysis which overlaps diaphysis medially. C. 61st day on experimental diets, 43rd day on 0.5 mg. ascorbic acid daily, day of death. No epiphyseal overlap because of increased proximal thickening of tibial diaphysis.

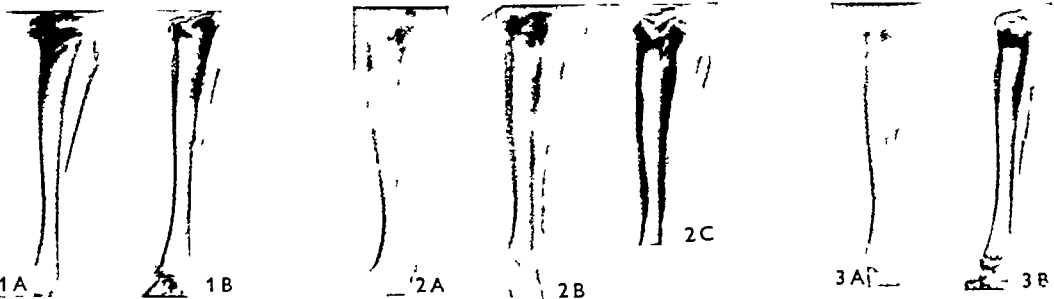
- Fig 3 From a partially vitamin C deficient guinea pig of Exp 5, group 4 A 27th day on experimental diets, 9th day on 0.5 mg ascorbic acid daily, day of operation Tibial epiphysis overlaps diaphysis medially, and on this side the diaphyseal wall has lost its curvature B 45th day of experimental diets, 27th day on 0.5 mg ascorbic acid daily, 19th day after operation Epiphysis detached from tibia, diaphyseal wall of tibia thickened near proximal end, no callus at fracture
- Fig 4 The knees and tibio fibulae of a normal animal
- Fig 5 The knees and tibio fibulae of a partially vitamin C deficient guinea pig, from Exp 7, group 1, 62nd day of experimental diets, 52nd day on 0.5 mg ascorbic acid daily, showing the bent knees, making the animal bow legged Contrast fig 4 Figs 4 and 5 are at the same magnification, and the scale between them is in centimetres
- Fig 6 From a normal guinea pig, frontal section of the proximal end of the tibia and fibula Azan $\times 9$
- Fig 7 From a partially vitamin C deficient guinea pig ('VA40') of Exp 6, group 4, 30th day of experimental diets, 14th day on 0.5 mg ascorbic acid daily, 6th day after operation Frontal section of proximal end of tibio fibula, showing an early stage in the development of new subperiosteal bone, periosteal cartilage, and endochondral and endosteal callus developing in the connective tissue which replaces the normal haematopoietic bone marrow Haematoxylin and eosin $\times 13$
- Fig 8 From the same partially vitamin C deficient guinea pig as fig 7, showing a microfracture at the top of the tibial diaphysis, above and to the right, periosteal cartilage Haematoxylin and eosin $\times 210$

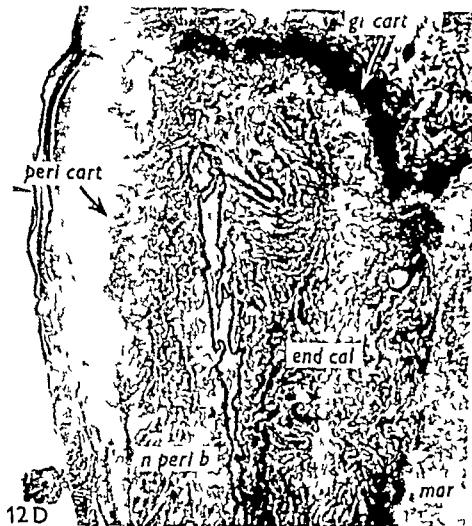
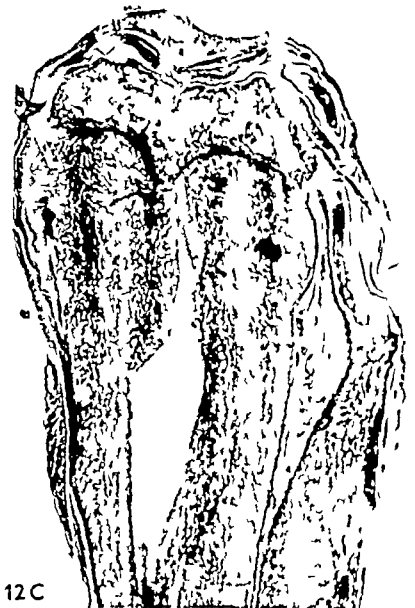
PLATE 2

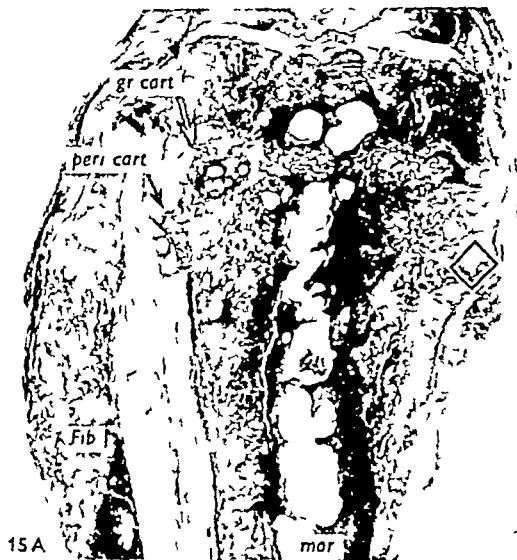
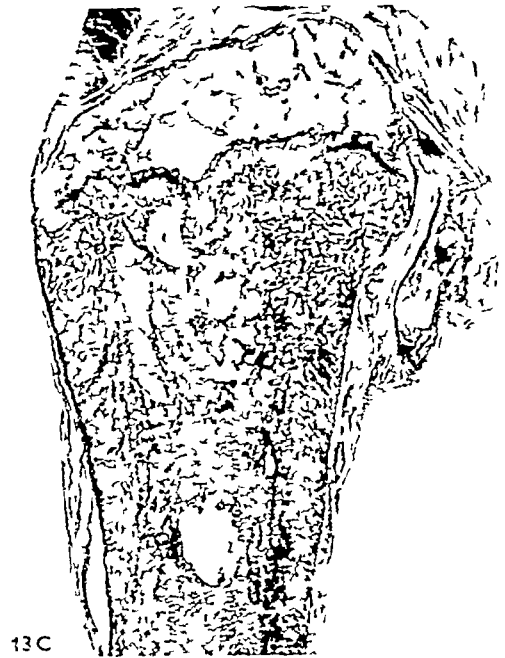
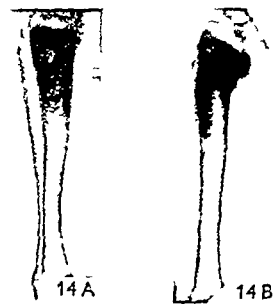
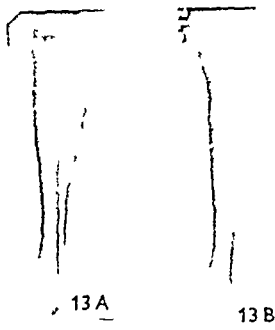
- Fig 9 From the same partially vitamin C deficient animal as fig 7 Frontal section of the proximal end of the fibular diaphysis, showing a microfracture and new subperiosteal bone developing in a widened cambium Haematoxylin and eosin $\times 62$
- Fig 10 From a guinea pig of Exp 8, group 2, allowed cabbage after 16 days total deprivation of vitamin C A 15th day of total deficiency Tibial epiphysis overlaps diaphysis medially B 64th day of experiment, 48th day on cabbage The tibial epiphysis shows a slight medial slope, and the diaphysis a thickening of its medial wall beginning a little below the growth cartilage C 66th day of experiment, 50th day on cabbage Frontal section of proximal end of tibio fibula, the tibial wall is thickened medially except just below the growth cartilage Haematoxylin and eosin $\times 7$
- Fig 11 From a partially vitamin C deficient guinea pig of Exp 5, group 4 47th day of experimental diets, 29th day on 0.5 mg ascorbic acid daily, 20th day after operation See also radiographs of this animal in Pl 1, fig 1 Part of frontal section of tibia, showing callus at top of the diaphysis, periosteal cartilage being resorbed, and new subperiosteal bone (low right) Haematoxylin and eosin $\times 17$
- Fig 12 From a partially vitamin C deficient guinea pig of Exp 5, group 4 A 27th day of experimental diets, 9th day on 0.5 mg ascorbic acid, day of operation Tibial epiphysis overlaps diaphysis on both sides, diaphysis lacks its normal proximal widening B-E 63rd day of experimental diets, 45th day on 0.5 mg ascorbic acid daily, 37th day after operation, day of death Great subperiosteal and endosteal thickening of diaphyseal wall, epiphysis no longer overlaps diaphysis C Frontal section of proximal end of tibia and fibula, showing immense thickening of diaphyseal walls and reduction of marrow cavity Haematoxylin and eosin $\times 7$ D Portion of medial wall of section near that shown in C, showing periosteal cartilage, the remains of the original diaphyseal wall outlined in black, and callus Haematoxylin and eosin $\times 17$ E Resembles D, but from the lateral wall, the dark area in the middle of the callus is unresorbed cartilage Haematoxylin and eosin $\times 17$

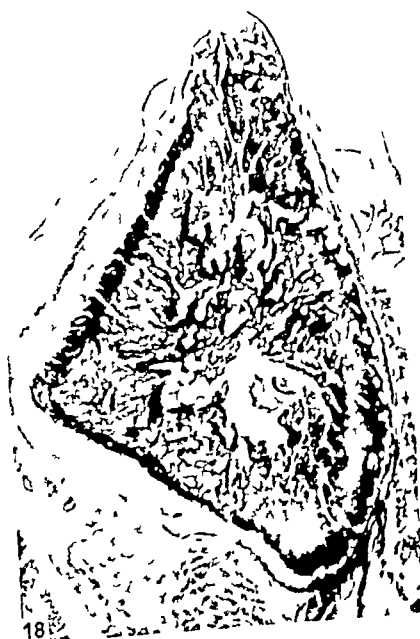
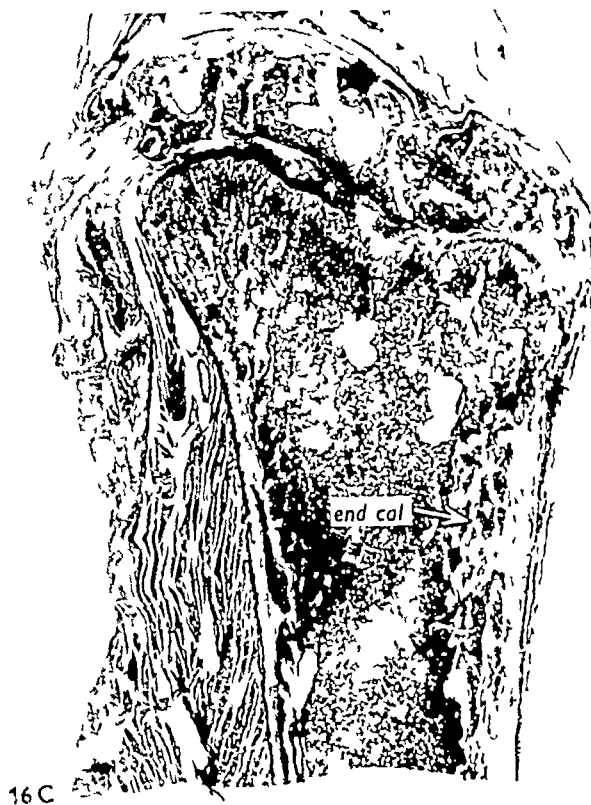
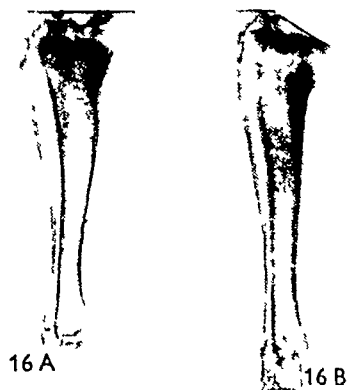
PLATE 3

- Fig 13 From a partially vitamin C deficient guinea pig of Exp 7, group 1 A 15th day of experimental diets, 5th day on 0.5 mg ascorbic acid daily Tibial epiphysis overlaps diaphysis medially B, C 62nd day of experimental diets, 52nd day on 0.5 mg ascorbic acid daily Medial slope of epiphysis, thickening of both diaphyseal walls C Haematoxylin and eosin $\times 7$
- Fig 14 The same animal as fig 13, photographs taken on same days as fig 13A and B, but of the other leg A Great medial overlap of tibial epiphysis B Extreme epiphyseal slope due to collapse of proximal end of diaphysis
- Fig 15 From a partially vitamin C deficient guinea pig of Exp 7, group 1 64th day of experimental diets, 54th day on 0.5 mg ascorbic acid daily A Frontal section of proximal ends of tibia and fibula, the tibial diaphysis shows immense thickening of the wall, reduction of the marrow cavity, and periosteal cartilage Haematoxylin and eosin $\times 6$ B The area in the black square in fig A more highly magnified, showing endochondral bone formation in the periosteal cartilage, and the









latter passing over into periosteum (from a section close in the series to A) Haematoxylin and eosin $\times 150$

PLATE 4

- Fig 16 From a guinea pig of Exp 8 group 2, allowed cabbage after 16 days total deprivation of vitamin C
 A 15th day of total deprivation of vitamin C, slight medial slope of tibial epiphysis B 64th day of experiment and 48th day on cabbage C Frontal section of proximal end of tibia, 66th day of experiment, 50th day on cabbage, showing medial slope of epiphysis and endosteal callus on the medial side, a little below the growth cartilage Haematoxylin and eosin $\times 7$
- Fig 17 From a normal guinea pig An oblique section across the tibial diaphysis near its proximal end Haematoxylin and eosin. $\times 12$
- Fig 18 From a partially vitamin C deficient guinea pig of Exp 5, group 3 121st day on experimental diets, 103rd day on 0.5 mg ascorbic acid daily The marrow cavity is almost obliterated by the endosteal callus Compare fig 17, a section which in position and orientation closely agrees with that here shown Haematoxylin and eosin $\times 115$

Abbreviations

<i>ct mar</i>	connective tissue of marrow ('Fasergerüst')
<i>end cal</i>	endosteal callus
<i>endo cal</i>	endochondral callus
<i>Fib</i>	fibula
<i>fib per</i>	fibrous layer of periosteum
<i>gr cart</i>	growth cartilage
<i>mar</i>	marrow
<i>n peri b</i>	new subperiosteal bone
<i>peri cart</i>	periosteal cartilage
<i>n b</i>	new bone

THE SIZE RELATIONSHIPS SUBSISTING BETWEEN BODY LENGTH, LIMBS AND JAWS IN MAN

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INTRODUCTION

For the purpose of an investigation into the growth of nerves in man, measurements were made on a large number (c 850) of male infants, children and adolescents living in London. The ages of the subjects ranged from 2 days to 20 years and their heights from 448 to 1870 mm. The examination of these data has revealed a number of facts of interest quite apart from those valuable for the original inquiry, and the present paper examines the relationships between the sizes of the limbs and jaw, and the total height of members of this group.

Many biologists have been interested in the relative sizes of parts of animals, and it has been maintained that the relationship between the size of a part and the size of the whole animal is best described by some kind of exponential (logarithmic) function. The outstanding examples of such work may be found in Huxley (1932) and Teissier (1937), while many other cases are described by Brody (1945). This kind of relationship is usually known as allometric or heterogonic. On the other hand, there is a considerable amount of evidence to show that in many cases the changes in size can be adequately described by a simple linear function, i.e. by stating that the size of the part is directly proportional to the total size of the animal.

MATERIAL AND METHODS

Since a wide range of ages had to be considered, four series of measurements were made. The first series was made on normal males of school-age attending London County Council schools in the north-west area of London. These boys were between the ages of 4 and 16 and were drawn from a uniform economic and social group. The second series was made on boys of 1-3 years. This series was carried out at day-nurseries in the same area from which the data covering the school-age boys were obtained, while the third series consisted of measurements made at University College Hospital on 50 newborn male infants, whose parents lived in similar areas. The fourth series of measurements was made on male University College students with an age range of 17-20. These students, although living in London at the time of the investigation, came from all parts of Great Britain and must have grown up in varied environments.

It will be seen that the whole series of these individuals may be considered to be a sample of the young male inhabitants of London, i.e. a sample from a highly cosmopolitan area.

The measurements were made in the following manner

(a) *Height*

A 2 m rule graduated in centimetres and millimetres, having metalled ends and placed vertically against a wall, door, etc., was used for measuring height. The subject stood with his back to this wall with heels, buttocks and occiput, touching the wall and eyes directed horizontally forwards.

(b) *Cubit*

A sliding caliper was used. In most cases the measurement was made on the right arm, exceptions being those cases in which there was a history, or other evidence, of injury to this arm. The subject was seated with his right forearm and outstretched hand placed prone on a table, the elbow being flexed to a right angle. The measurement was then made from the posterior surface of the elbow over the olecranon process, to the tip of the middle finger.

(c) '*Leg*'

The subject was seated with the right shoe removed and knee flexed to a right angle while the distance from the upper edge of the patella to the lower surface of the heel was measured with a sliding caliper. The word 'leg', as used in this paper, differs from standard anatomical usage in that the lower end of the femur, talus and os calcis are included.

(d) *Mandible*

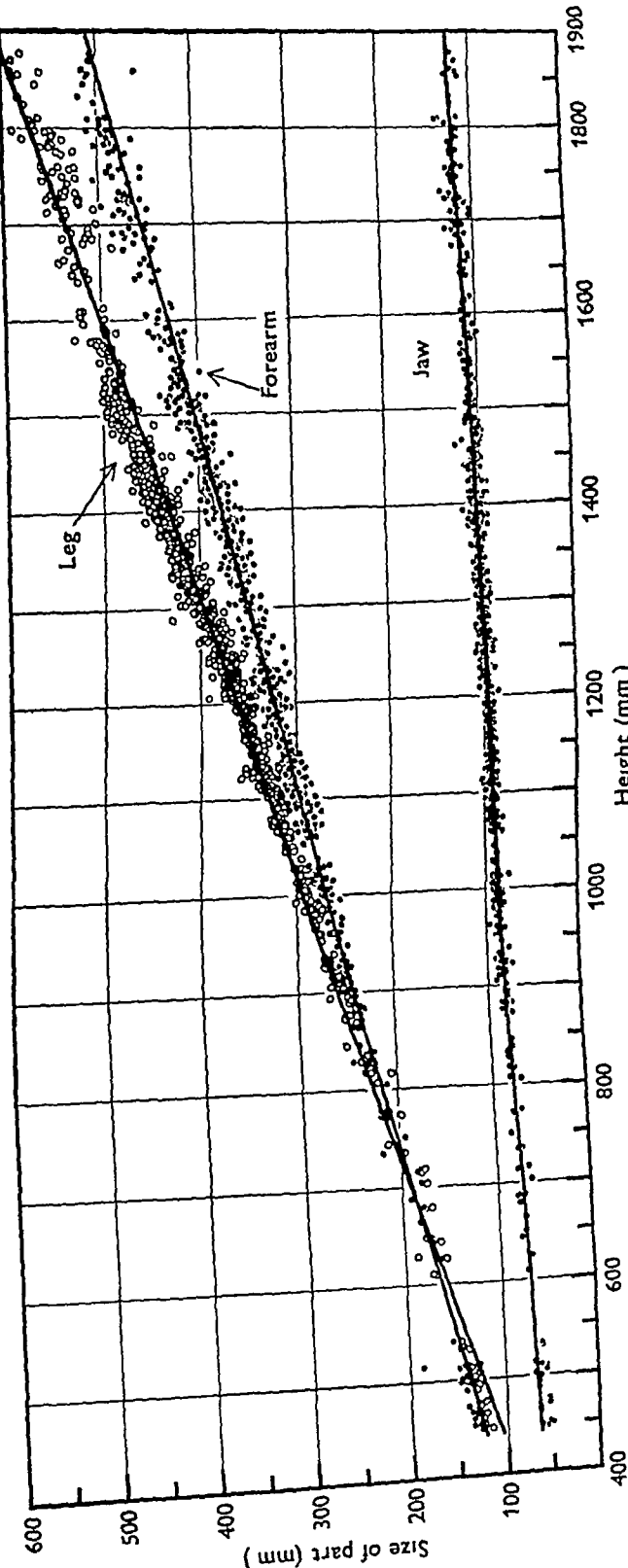
The measurement chosen as an index of mandibular size was the perpendicular distance between the bi-auricular axis and the symphysis menti. A 'head-spanner' was used for this measurement (see Pl. 1). This spanner consists of two adjustable wooden rods *A* and *B* with tapering ends attached to the frame as shown. The ends are made to fit snugly but not tightly into the external auditory meatus. A third adjustable rod *C*, calibrated so that direct readings can be made from it, makes contact with the symphysis menti and the perpendicular distance between the line *AB* and *C* is shown at *D* (on rod *C*).

Some variations in technique were used for measuring the infants. For height, the crown-heel length in the supine position was taken, measured by a large sliding caliper. Mandibular size was measured as before, and a small sliding caliper was used for the cubit and leg measurements.

ANALYSIS OF THE DATA

The data were plotted on squared paper and the result is reproduced in Text-fig. 1. It is obvious that some kind of linear hypothesis is suggested, since there appears to be no indication of curvature, moreover, there is no need to postulate any kind of polynomial or exponential relationship between the variables.

Denoting the values of the body height by x and those of the length of the part (limb or jaw) by y we may consider the linear regression of y on x . We assume that the x values are known accurately and that for any given x the corresponding values of y are normally distributed about a mean $a + bx$ with a standard deviation which must be estimated from the data. These assumptions are, of course, those



Text fig 1 Plotted points of all the observations and the regression lines calculated from the data

usually made for fitting linear regressions, the calculations were made from ungrouped data. One set of data is less satisfactory than the other two since it is clear that any change in length of leg must lead to a change in total height and, consequently, there is a kind of spurious correlation between the measurements of leg and those of height. This, however, is unavoidable and the estimates of relative size are unaffected.

Table 1 *The values of the parameters to be inserted into the regression equation $y = a + bx$. The values of $1.96\sqrt{V}$ are used to determine confidence limits as described in the text*

Part (y)	a	b	\sqrt{V}	$1.96\sqrt{V}$
Arm	-0.30	0.27	8.975	17.59
Jaw	39.80	0.045	4.789	9.39
Leg	-50.31	0.34	14.430	28.28

Table 2 *The values of certain computed functions which present the information necessary for most statistical investigations on the data*

Part (y)	Number measured	Mean height (\bar{x})	Mean y (\bar{y})	Standard error of b	$\Sigma(x - \bar{x})^2$	$\Sigma(y - \bar{y})^2$	$\Sigma(x - \bar{x})(y - \bar{y})$	Correlation coefficient	Residual sum of squares
Arm	847	1246.77	335.89	0.001	75 864 940	5 584 438	20 457 214	0.99	68 069 244
Jaw	842	1249.07	95.69	0.0006	75 050 582	169 390	3 356 675	0.89	16 263 618
Leg	847	1246.77	379.35	0.0016	75 864 940	9 186 006	26 144 741	0.98	175 953 053

The individual measurements are not presented here from considerations of space, but they have been deposited and may be consulted at the Thane Library, University College, London. Summaries of the information derived from the data are shown in the Tables. Table 1 gives the values of certain parameters which will be discussed later, while Table 2 contains the results of certain computations which may be useful to other investigators.

It is quite clear from the values of the residual sums of squares that the regression coefficients are significant, and the high values of the correlation coefficients indicate the suitability of a linear hypothesis. Moreover, these coefficients show that the regression lines of y on x and x on y are practically coincident.

The lines have been calculated in the form

$$y = a + bx, \quad (1)$$

where y = length of part (mm), x = height (mm). If the parameter values are substituted into equation (1), we find the following equations for the regression lines

- (a) Arm/height $y = -0.30 + 0.27x$
- (b) Jaw/height $y = 39.80 + 0.045x$
- (c) Leg/height $y = -50.31 + 0.34x$

The lines have been drawn to the plots of the data in Text-fig. 1.

The values for a have no biological significance since they are formed partly from means and partly from regression coefficients, but the values for b are measures of the change in lengths of the limbs and jaw for any given change in height. If the height increases by 10 cm then the arm increases by 2.7 cm, the leg by 3.4 cm and the jaw by only 0.45 cm; the leg shows the largest relative increase, the arm a some-

what smaller increase, while the change in the length of jaw is very small indeed. In a certain sense, these values of b may be considered as measures of relative growth.

Furthermore, data of this kind provide norms which may make it possible to detect early stages of faulty skeletal development. At present, reliance has to be placed on subjective judgements, and the physician may have to wait some time before the skeletal defect is sufficiently obvious. With data of the kind used in this paper it is a simple matter to test if the skeletal development of an individual lies within the usual range of variation. The method to be used is outlined below, and a particular numerical case is worked out as an example.

The equations enable us to find the size which is to be expected for one or all of these parts for a given height. It is only necessary to substitute the value of the height for x in the appropriate equation and then the value of y (size of the part) is easily calculated. Such an estimate is of limited value since the equation has only been fitted to a sample of the population and no matter how large the sample size, any estimates of values of parameters for the whole population will be subject to error. It is possible, however, to specify boundaries within which the size of the part may be expected to lie. Such boundaries are frequently referred to as confidence intervals or confidence limits and were discussed by Neyman (1937), the theory of such intervals has also been explained by Wilks (1944) and Cramer (1946).

In order to find such confidence limits we must first specify the proportion of cases in which we hope our statement to be correct, e.g. 95 or 99%. Then we can calculate two numbers θ_1 and θ_2 for every x , such that if we make the statement that y lies between θ_1 and θ_2 , we shall be correct in 95% (or 99%) of the cases in the long run.

The group of all the individuals who were measured comprises a sample drawn from the population of London inhabitants and the conclusions may be considered to be valid for this population providing that no values of height outside the range considered are involved. The conclusions would not necessarily apply to the inhabitants of another part of the country since the sample could not be assumed to be representative without further investigation.

If the sample upon which the estimates are based is small, the formulae for finding the confidence limits are a little more complicated,* but in the present case we may use the expression $Y = y \pm 1.96\sqrt{V}$, where y is the value found from the regression equation and V is the estimate of variance found from the residual sum of squares. The values of $1.96\sqrt{V}$ are given in Table 1.

By way of example, let us assume that we wish to know the length of arm to be expected for an individual 1747 mm high. We wish to answer a question of the form 'Is it reasonable to expect a normal Londoner who is 1747 mm high to have an arm 500 mm long?' The regression equation for the arm is

$$y = -0.30 + 0.27x,$$

* The exact confidence limits for an individual are given by

$$Y = y \pm 1.96 \sqrt{V \left(1 + \frac{1}{n} + \frac{(x - \bar{x})^2}{\sum (x - \bar{x})^2} \right)}$$

In the case considered the last two terms may be ignored

and the substitution of 1747 for x in this expression leads to a value of 470.78 for y . The value for $1.96\sqrt{V}$ given in Table 1 is 17.59 and the confidence limits for Y are

$$Y = 470.78 \pm 17.59,$$

and, with 95% confidence, we can assert that for a height of 1747 mm, the arm length lies between 453 and 488 mm. Consequently, we should consider an arm length of 500 mm as 'abnormal' for this height.

DISCUSSION

The study of the relative growth of parts of organisms became more widespread after the publication of Huxley's book on *Problems of Relative Growth* (1932). The author showed that when the logarithms of the weights or dimensions of parts of an organism were plotted against those of the whole animal, the plotted points often appeared to fall about a straight line. If this were the case, it would imply an exponential or compound interest relationship between the variables of the form $y = bx^{\alpha}$, when the value of α differed from unity, such growth was described as heterogonic or allometric.

The results for many sets of data appeared to be convincing, but cases were frequently found when it was necessary to draw two or more straight lines in order to fit the logarithmic plots by straight lines. Moreover, certain observers, including D'Arcy Thompson (1942), found that a simple linear relationship was often adequate to describe the data, and since there is no *a priori* reason to prefer the allometric form and if the data show no curvilinear trend, then there is no justification for departing from an hypothesis of direct proportionality. The numerous theoretical objections to the allometric formula have been discussed in the essays by Reeve and Huxley and by Richards and Kavanagh in the volume edited by Le Gros Clark & Medawar (1945). The problems involved in fitting the allometric curve have been discussed elsewhere (Sholl, 1948).

It may be pointed out that it is sometimes thought that some justification for the allometry concept may be derived from the assumption that living cells themselves produce living cells which reproduce in their turn, i.e. a compound interest system. Reeve and Huxley point out that bone does not grow interstitially and there is little reason to assume that limbs would show this type of growth. There is a considerable literature on the subject and full bibliographies may be found in the essays cited.

The sets of data considered in this paper do not show any kind of allometric relation, the size of the part is directly proportional to the total size from the time of birth to maturity. It is true that, outside the range we have considered, e.g. in foetal life and during periods of life in which a more unrestricted type of growth prevailed, the heterogonic relationship might well hold. It is, however, interesting to note that during infancy and at puberty there is no evidence of such a tendency. Not only do the plotted points show no indication of any curvature but the high values of the correlation coefficients indicate that the linear hypothesis is justified, in fact, there is no reason to fit any kind of exponential curve. Although the sampling was not from a homogeneous group, the large number of observations with so small

a scatter indicates that the calculated lines do represent a mean tendency about which the values of the sizes of the part vary

It is, of course, obvious that such lines give no measure of any growth rate, since such rates must be concerned with the change in size with respect to time. Time has not been considered in this investigation, and consequently, the gradients of the lines only give information as to change in size of the part in relation to change in size of the whole individual

SUMMARY

1 Measurements of limbs, jaws, and total height were made on 795 male human beings from various parts of the London area. The ages ranged from the newly born (2 days old) to 20 years old, while the height ranged from 448 to 1870 mm

2 The regression of the length of the part on the total body length is linear, consequently there is no evidence for allometric growth at any period between infancy and maturity

3 Sufficient data are published and methods are given for finding the confidence limits between which the size of the part may be expected to lie when any particular value of the total length is considered. The application of this method as an aid to the diagnosis of pathological states is shown

We wish to thank Prof J Z Young for the advice and encouragement which he has freely given during the course of this work and we are most grateful to the London County Council for their co-operation in this work and, particularly, to Dr J N Dobbie, Senior Medical Officer of Health to the London County Council, and Dr G W Pritchett, the Medical Officer for the north-west region. We must also thank the Head Teachers of the various schools for their co-operation and Prof F J Brown, lately Professor of Obstetrics in University College Hospital, for permission to make measurements on the newborn infants, Sister Edwards gave us expert assistance

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THE PROJECTION OF THE CAUDAL SEGMENTS OF THE SPINAL CORD TO THE LINGULA IN THE SPIDER MONKEY*

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INTRODUCTION

In investigating the degree to which the organization of the central nervous system is influenced by the presence of a prehensile tail in the spider monkey, we found that the eight caudal segments of the spinal cord which are exclusively concerned with the tail (Chang & Ruch, 1947*a*) are projected to a particular cell group of dorsal column nuclei (Chang & Ruch, 1947*b*) and a particular region in the thalamus (Chang & Ruch, 1947*c*). Most interesting of all is the finding reported below, i.e. that the projection of these terminal segments of the spinal cord is to the foremost part of the anterior lobe of the cerebellum.

This finding, we believe, offers anatomical support for the theory of topographical representation of the cerebellum as advanced in recent years by physiologists, namely that the tail, leg, arm and face are represented in the anterior lobe anteroposteriorly in that order.

EXPERIMENTAL RESULTS

*Experiment 1 Spider monkey series no 13, adult male Spinal transection at Ca₁
Operated 1 Sept 1944 Sacrificed 1 Oct 1944 Swank-Davenport preparation*

A compact bundle of degenerated fibres is present in the periphery of the antero-lateral funiculus just above the lesion. The ventral spinocerebellar tract is distinguished from the other components of Gowers' fasciculus by its comparatively small size, by the closer aggregation of the fibres, and by its ventrolateral position which is maintained throughout the cord.

In the lower medulla, the degenerated fibres in the ventrolateral fasciculus of Gowers can be followed to the region of the nucleus lateralis medullaris where they occupy a triangular area between the inferior olive and the descending trigeminal tract. Here, some of the fibres, mostly those of large size, veer medialward to the reticular formation dorsal to the inferior olive and the remaining fibres proceed farther cephalad. While ascending to the level of the caudal end of the pons, the cerebellar fibres turn dorsally and occupy the dorsolateral margin of the medulla, arching around the brachium conjunctivum. Some of them turn backwards, passing along the medial wall of the superior cerebellar peduncle and the others course through the anterior medullary velum to the cerebellum. In transverse sections

* This paper constitutes a part of a dissertation 'Segmentation, lamination and topographical projection of the nervous system with special reference to the tail of *Ateles*' presented in 1946 by H. T. C. to the faculty of the Graduate School of Yale University in candidacy for the Degree of Doctor of Philosophy.

taken at the level of the exit of the vagus nerve, the spinocerebellar fibres appear in the region between the inferior olive and the descending trigeminal tract, and a few degenerated fibres are seen in the median plane of the cerebellum between the nuclei fastigii. The latter fibres are considered to be those passing to the uvula and pyramis.

In the sections taken at the level of the eighth nerve and also at the anterior end of the dentate nucleus of the cerebellum, the backward course of the spinocerebellar fibres along the medial border of the superior cerebellar peduncle is found. Here, the degenerated fibres are seen distributed along the median plane of the anterior lobe of the cerebellum.

From sections taken at the level of the pons, the dorsalward shifting of the ventral spinocerebellar tract and the termination of degenerated fibres in the lingula and the lobulus centralis are shown.

The degenerated fibres in the anterior lobe are strictly confined to the median plane.

Experiment 2 Spider monkey series no 14, adult, female Left spinal hemisection at Ca₁. Operated on 8 Sept 1944. Sacrificed 26 Sept 1944. Swank-Davenport preparation.

The degenerated fibres resulting from the left hemisection of the spinal cord at the first caudal segment are present in the fasciculus of Gowers on both sides, but those on the right side, which originate from cells at the site of the lesion on the left, are much fewer in number.

At the level of the pons, the cerebellar components of Gowers' fasciculus shift dorsally to the dorsolateral border of the brachium conjunctivum, curving around the dorsal aspect and then the medial aspect of the superior peduncle, and finally entering the cerebellum.

The distribution of the degenerated fibres in the cerebellum is similar to that seen in Exp 1. In addition, the number of degenerated fibres to the left of the median plane of the anterior lobe is much greater than on the right side. This seems to be an indication of the predominant ipsilateral termination of the ventral spinocerebellar tract. The fibres cross in the great anterior cerebellar commissure. No degeneration products are found in the lateral extensions of the anterior lobe, in the paraflocculus, or in any other part of the cerebellar cortex.

Experiment 3 Spider monkey series no 17, 1-year-old female Spinal transection at Ca₁. Operated on 28 Nov 1944. Sacrificed 11 Dec 1944. Swank-Davenport preparation.

The medulla and the cerebellum were cut in sagittal sections in order to gain a better orientation of the degenerated fibres in the cerebellum. In the sections from the most lateral part of the medulla, the course of the degenerated fibres in the fasciculus of Gowers is distinctly stained. At the caudal end of the medulla, the degenerated fibres present a wavy appearance. They weave through the cell groups of the nucleus lateralis medullaris, which probably receive no terminations of the degenerated fibres. Farther cephalad, the degenerated fibres are grouped in a bundle passing over the dorsal surface of the inferior olive. From the caudal end of the pons onwards the tract gradually turns more dorsally. At a mid-pontine level the fibres are divided into two parts, the smaller ventral part proceeds farther forwards and

some of its fibres are gradually dispersed and probably terminate in the reticular formation. The large dorsal portion goes towards the floor of the fourth ventricle, beneath which the degenerated fibres are further divided into two subgroups. One group turns backwards to the cerebellum, chiefly by way of the anterior medullary velum and the other group, including the spinotectal and spinothalamic fibres, continues on to the midbrain and thalamus. The distribution of the degenerated fibres is shown in a sagittal section of the cerebellum (Pl 1, fig 1). Degenerated fibres coming from the anterior medullary velum are shown. Degeneration products are abundant in the lingula. The degeneration in the lobulus centralis (lobulus 2 of Bolk) is apparently heavier than in the lobulus 3 of Bolk. Only a few fibres enter the anterior margin of the large medullary lamina in the culmen. In the posterior part of the cerebellum, i.e., posterior to the fissura prima, only the upper and lower lips of the fissura secunda receive degenerated fibres from the ventral spinocerebellar tract (Pl 1, fig 2). The posterior part of the culmen, the lobus simplex, the declivus and the nodulus are virtually free of degeneration. All the degeneration products are confined to the white matter.

To summarize In the spider monkey the ventral spinocerebellar tract can be found in the lowest segments of the spinal cord. In their course through the spinal cord and medulla, the fibres in this tract always occupy the most peripheral region and are immediately external to, and partly mingled with, the spinothalamic fibres. They enter the cerebellum by way of the anterior medullary velum and the anterodorsal aspect of the superior cerebellar peduncle. On reaching the cerebellum some fibres cross through the rostral part of the great anterior cerebellar commissure and both the crossed and uncrossed fibres run to the lingula, the lobulus centralis and the anterior folia of the culmen. No fibres go to the posterior culmen or to any part posterior to the fissura prima except an isolated bundle to the walls of the fissura secunda. After a transection of the spinal cord at C_4 , the severest degeneration was found in the lingula and the anterior folia of the lobulus centralis, only a few fibres could be traced to the anterior part of the culmen and the walls of the fissura secunda.

DISCUSSION

In all of the three experiments described above, the lesions were made at the first caudal segments. Because the dorsal spinocerebellar tract in the caudal cord is not prominent and cannot be easily traced to the higher levels of the brain, the degenerations observed in the cerebellum are chiefly those contributed by the ventral spinocerebellar tract.

In 1909 McNalty & Horsley, working chiefly on macaques, concluded that fibres of the ventral spinocerebellar tract terminate in the whole anterior lobe of the cerebellum *except the lingula*. However, the termination of the ventral spinocerebellar tract in the lingula has been demonstrated since then by Ingvar (1918), and Beck (1927) in the cat, and Anderson (1943) in the rat. McNalty & Horsley's failure to find termination of spinocerebellar fibres in the lingula might be associated with the poorly developed caudal segments in the macaque.

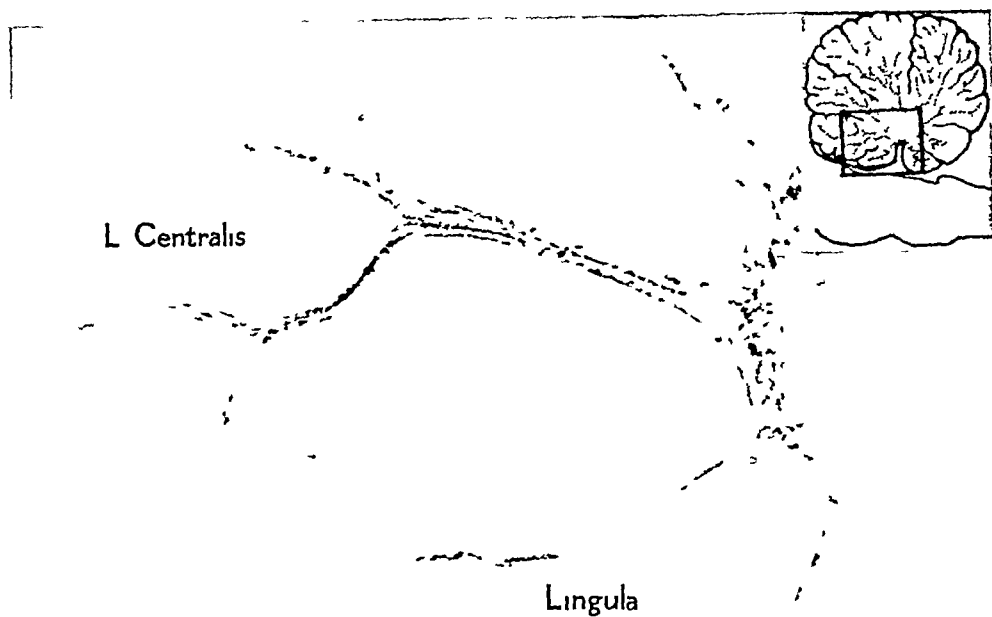
It is suggestive that such a large number of ventral spinocerebellar fibres should terminate in the lingula of the spider monkey, which has a very highly developed tail, and that the amount of degeneration in the lingula after a cervical anterolateral

cordotomy is not significantly greater than that resulting from a caudal transection. It is evident that the lingula receives fibres mainly from the caudal segments of the cord which are largely related to the prehensile tail in this animal. Our findings afford an anatomical basis for the recent physiological findings of Adrian (1943), Snider & Stowell (1944), Dow & Anderson (1942), and Hampson, Harrison & Woolsey (1945). Adrian demonstrated by electrical methods that sensory impulses from the lower part of the body are projected to the rostral end of the anterior lobe of the cerebellum and those from the upper part of the body, to the posterior end. Snider & Stowell found that the somatotopic projection on to the cerebellum is in the same order as that postulated by Adrian. Hampson *et al* described a sequence in the somatotopic localization of motor function in the cerebellum, with the tail end at the lingula. More recently (1946) they reported that the strongest motor effects in the tail were obtained by electrical stimulation of this particular region. It is, therefore, probable that both afferent and efferent cerebellar mechanisms for the tail are situated in the lingula. Another clue to the functional significance of the lingula is its special development in the sea mammals, in which the tail is a well-developed propulsive and 'steering' organ whereas the limbs are retrogressive.

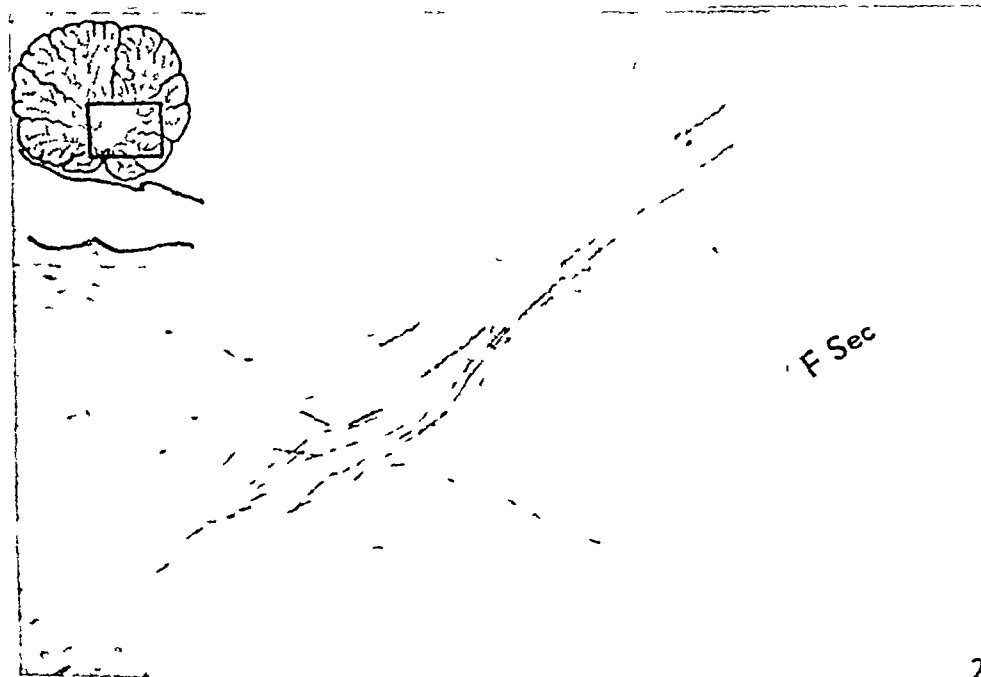
The cerebellum of the spider monkey is characterized by a large and laterally expanded paraflocculus which Bolk (1906), from a morphological and phylogenetic point of view, related to the development of the tail in this animal. Bolk's correlation of the paraflocculus with the tail is probably correct, although the theoretical basis of his statement is no longer valid. He regarded the tail of the spider monkey as an organ of balance, functionally similar to the tail of whales. This is, however, not true. His second assumption that the paraflocculus is concerned with body equilibrium is unlikely, in view of the present knowledge of the cerebellum. According to the analysis by Larsell (1947), the paraflocculus belongs to the corpus cerebelli and is distinct ontogenetically from the flocculus. It has no fibre connexions with the vestibular apparatus. Thus, the explanation of the development and the bulk of the paraflocculus in tailed animals has to be found in a different direction. As first demonstrated by Ingvar (1918), the paraflocculus has afferent connexions from the spinal cord. In our material, the degenerated fibres could not be followed as far as the paraflocculus, but could be traced to the upper and lower lips of the fissura secunda, i.e., to the inferior part of the pyramis and the superior part of the uvula. It should be noted in our Pl. 1, fig. 2 that the degenerated fibres are sharply confined to the lower half of the medullary lamina of the pyramis and the upper half of the arbor vitae of the uvula. This distribution of the afferent fibres from the caudal region of the cord is perhaps a clue to the function of the paraflocculus, since according to Larsell & Dow (1935) the paraflocculus is derived from the pyramis and uvula.

SUMMARY

The degenerated ventral spinocerebellar fibres, after spinal transection or hemisection at Ca_1 in three spider monkeys, have been traced to the anterior part of the anterior lobe of the cerebellum, with the heaviest degeneration in the lingula and the anterior folia of the lobulus centralis. No degenerated fibres go to the posterior folia of the culmen or to any part posterior to the fissura prima except a few fibres to the walls of the fissura secunda.



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EXPLANATION OF PLATE

- Fig 1 Photomicrograph from a sagittal section of the cerebellum, illustrating the fibre degeneration in the lingula and the anterior folia of the lobulus centralis 13 days after spinal transection at C_4 . The square in the inset figure indicates the location of the section photographed Spider monkey no 17
- Fig 2 Photomicrograph from a sagittal section of the cerebellum, showing the distribution of degenerated fibres in the medullary laminae above and below the fissura secunda Spider monkey no 17

TWO EARLY HUMAN EMBRYOS

By W R M MORTON

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The two embryos described here were given to me by Prof J H Biggart, who had recovered them from curettage material removed by Prof C H G Macafee, I have named the older specimen the Biggart Ovum and the younger the Macafee Ovum. The material was fixed in formol-saline and later transferred to formol-Zenker, it was embedded in paraffin, cut at 6μ and stained with haematoxylin and eosin.

THE BIGGART OVUM

The Biggart ovum was recovered from a woman aged 24 years, married 7 years, one child 4 years 8 months, no miscarriages, menstruation began at 15, 7-day loss recurring very regularly every 21 days, last menstruation stated to have ended 29 June, admitted to hospital 12 July and curetted 16 July, coitus said to have occurred only once between the end of menstruation and admission, probably on 2 July and certainly between 1 and 5 July.

The specimen consists of a piece of endometrium in which the ovum is very superficially implanted (Pl 1, fig 1). Examination shows the preservation to be good and that only some peripheral villous material and one section containing embryonic material proper are missing, but some of the sections are a little distorted. It was found to be cut almost vertically to the embryonic shield and obliquely at 13° to its sagittal plane. The primary measurements in millimetres of the ovum and its parts are as follows:

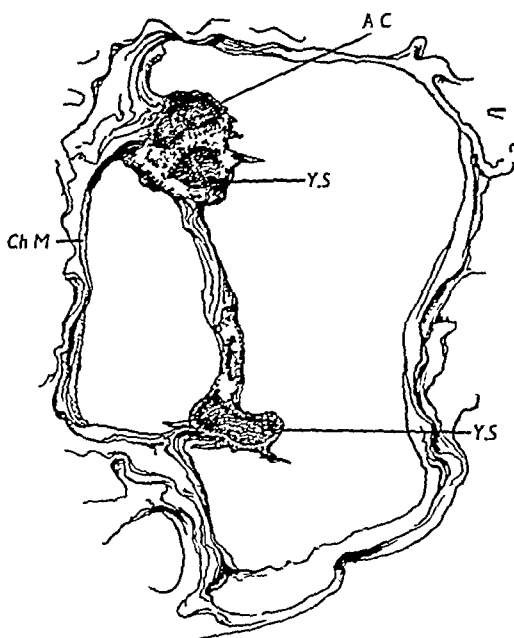
	Antero posterior	Vertical	Transverse
Trophoblast, external, including the villi	2.53	2.10	1.68
Trophoblast cavity	1.53	1.05	0.83
Shield ectoderm	0.27*	0.027	0.16
Amniotic cavity	0.22	0.10	0.16
Yolk sac cavity	0.19	0.82	0.18

* Taken diagonally from the graphic reconstruction.

The endometrium shows early decidual reaction and contains peripheral syncytium. Beyond the limits of the trophoblastic shell, there is no zone of necrosis. The glands are dilated and in the saw-tooth phase, they contain extravasated blood and secretion. Large blood sinuses are present beneath the ovum and seem to have limited the villus formation over one part of the vesicle. The site of entry is wide and filled with the thin ab-embryonic wall of the chorionic vesicle covered by a thin layer of schlusascoagulum, there is no operculum.

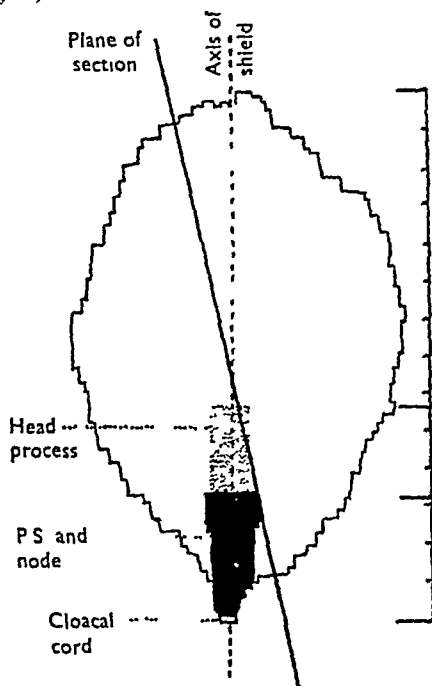
The trophoblast, which is lined with a chorionic mesoderm, consists of cytotrophoblast and syncytiotrophoblast, and villi are present over most of it, but are absent over the superficial surface and over one part of the deep surface near a large venous sinus (Pl 1, fig 1). Many of the villi contain a mesodermal core and some have begun to branch.

The chorionic vesicle contains a granular coagulum surrounding the embryonic rudiment, which consists of an embryonic shield, a small amniotic cavity, and a larger yolk-sac which is continued by a long narrow tubular duct into a large irregular expansion (Text-fig 1) The embryonic rudiment is joined to the chorionic mesoderm first by a broad connecting stalk attached to the amnion, and secondly by mesoderm at the distal expansion of the yolk-sac (Text-fig 1)



Text-fig 1

Text-fig 1 Drawing of wax model cut to show the relations of the embryonic mass A C Amniotic cavity
Ch M Chorionic mesoderm Y S Yolk sac



Text-fig 2

Text-fig 2 Scale diagram of surface view of the embryonic shield

The embryonic shield is a broad oval in dorsal view and is not quite symmetrical about its long axis (Text-fig 2) It is slightly convex towards the amniotic cavity. Its margins are continuous with the thinner amniotic ectoderm, the transition being clearly marked (Pl 2, fig 4) The shield ectoderm consists of high columnar cells which in places appear to be stratified, but this is due to the obliquity of the section, those of the central area are larger than those nearer the margin and are more vacuolated and granular. At the posterior end of the shield and reaching its margin there is a median linear strip of lighter more loosely arranged polyhedral cells (Pl 2, fig 5), the strip extends over six sections and as measured by reconstruction is 0.059 mm long and 0.036 mm broad, that is, it is a little more than one-fifth of the length of the shield (Text-fig 2) The cells forming it are continuous in front and at the sides with the general shield ectoderm, and below they are in close contact with the gut endoderm but can be distinguished from it, there can be little doubt that it is the primitive node and streak.

The main interest of the specimen lies in the condition of the yolk-sac. The form of its three parts is shown in Text-fig 1. The wall of the whole of the proximal part consists of a single layer of round or oval cells much smaller than the overlying ectodermal cells and easily distinguishable from them. Cells of the same kind bound a small wide diverticulum which passes backwards, below the level of the connecting stalk, under the posterior end of the shield, which may represent the allantoic diverticulum or may be a part of the expanding yolk-sac which is later incorporated in it as described by Florian (1930). The cells lining the duct part of the sac are cuboidal in form, have large round nuclei and are arranged in a single continuous layer (Pl 1, fig 2), on the whole they are slightly larger than the cells of the proximal part. The cells lining the distal expansion (Pl 1, fig 3) gradually change from cuboidal above to thin flattened squamous-like cells below, and over the greater part are easily distinguishable from the covering layer of mesoderm, but the central part of the ab-embryonic wall is very thin and consists of a single layer of very flattened cells the nature of which, endoderm or mesoderm, I am unable to decide.

The mesoderm covers the duct and the proximal part of the yolk-sac and is a continuous layer over the amnion, it forms the broad irregular connecting stalk. There is a small amount of intra-embryonic mesoderm in the peripheral rim of the embryonic shield which is directly continuous with the extra-embryonic mesoderm, and in the angle between the anterior end of the shield and the yolk-sac there is a more loosely arranged mass of mesoderm which may well be the protocardiac area. There are a few projections, with indications of cavity formation, in the mesoderm of the proximal yolk-sac and cavities in the chorionic mesoderm, especially in the region of the connecting stalk (Pl 2, figs 4, 5), these have been taken to be the first evidences of angiogenesis.

Discussion

The next menstruation, from the history given, should have begun on 14 July, and, taking ovulation to have occurred 14 ± 2 days before this, the single coitus probably on 2 July, falls within the probable period of ovulation 28 June to 2 July, the probable corial age is thus $13\frac{1}{2}$ days. The general dimensions, structure and differentiation of the embryo, and the appearances of the decidua agree with this estimate and place the ovum in Streeter's (1942) Group VII, and in comparison with known ova it appears to be more developed than embryo No 7801 ($13\frac{1}{2}$ days) of Heuser, Rock and Heitig (1945), of about the same stage as the Yale embryo (14 days) (Ramsey, 1938), and younger than the Falkner ovum (15–17 days) (Martin & Falkner, 1938). The interest lies in the hitherto undescribed form of the yolk-sac.

The presence of a duct-like process leading distally from the yolk-sac and connected to the chorionic mesoderm was described by Bryce (1924) in the ovum T B 2 and he noted that similar formations were present in other ova (Schlagenhauser and Verocay, Fetzner, Strahl-Benecke) and that in the Frassi ovum there is a detached vesicle lined with endoderm which is connected to the yolk-sac by mesoderm. Heuser *et al* (1945) describe in embryo No 7801 several large vesicles in the peripheral part of the chorionic cavity, the walls of which are for the most part composed of a single layer of flat squamous-like cells, but occasionally containing patches of cuboidal cells, the largest vesicle is attached by a faint strand of cytoplasm, which itself contains

a vacuolated group of cells, to the yolk-sac. The same authors also describe in embryo No 7802 (16½ days) a tapering funnel-shaped ventral process of the yolk-sac the mesodermal layer of which can be traced, as a fine filament, across the chorionic cavity to the mesoderm of the opposite side. Heuser & Wislocki (1935) have shown in the sloth (*Bradypus griseus*) that in early stages there is a chorionic attachment of the ab-embryonic wall of the yolk-sac and the formation of two yolk-sac vesicles connected, for a time, by a narrow duct. Later the connecting duct disappears, leaving a definitive yolk-sac under the shield and an endoderm-lined cyst-like cavity attached to the ab-embryonic wall of the chorion. Martin & Falkner (1938) state that the yolk-sac of the Falkner ovum is partially divided into two by a septum, and that the endoderm lining the cavity of the yolk-sac dorsal to the septum is cuboidal, and the endoderm of the part ventral to it is thin, flattened and smooth, except at the extreme ventral pole where it also is cuboidal. They also give details of a similar partial subdivision of the yolk-sac in a 15-day-old macaque embryo (No C 571) and state that the differences of the two types of endoderm are more evident than in the human. They offer two explanations for the occurrence of the divisions of the yolk-sac. They state first (p. 257) that the grooves (external to parts of the septum) probably represent the earliest sign of the folding-off of the embryo, and that they separate the gut endoderm dorsally from the yolk-sac vesicle endoderm ventrally, as suggested by Streeter. On p. 266 they state that if the process of taking up evaginations of the yolk-sac 'had gone a little further the septum partially dividing this region of the yolk-sac would have disappeared and the part of the allantois situated in the caudal wall of the yolk-sac' (i.e. below the septum) 'would have been incorporated in the yolk-sac cavity'. This implies that some of the thin flattened endoderm assigned earlier to the yolk-sac vesicle becomes transformed into cuboidal gut endoderm. It is apparent, however, from a study of the Falkner ovum, that the partial division of the yolk-sac seen in it is of a different nature from that seen in the present specimen. The endoderm cells lining the duct part of the yolk-sac of the Biggart ovum are slightly larger than the cells lying below the shield ectoderm and lining the proximal part of the yolk-sac cavity. It is only in the distal expansion of the yolk-sac that the lining cells become thin, flat and smooth, and in part of the ab-embryonic wall they are even more flattened and are either endoderm alone or mesoderm (Pl. 1, fig. 3). The appearances in the Biggart ovum are much more suggestive of the condition found in the 12-somite sloth. This stage could easily lead to the condition found in the 13½-day human (No 7801) by Heuser *et al.* or to that in the 38-somite sloth embryo. The appearance of the endoderm cells of the ab-embryonic parts of the vesicle possibly is due to a slow rate or absence of division of the cells, or perhaps to a falling off of the activity gradient and to relatively poorer nutrition at the ab-embryonic region. The 'stretching-out' of the yolk-sac by the expansion of the whole chorionic vesicle, to which the yolk-sac is attached, probably is the direct cause of the thinning of the distal yolk-sac cells, the duct cells remaining, or becoming, cuboidal because of the small diameter of the duct relative to the rest of the yolk-sac.

The origin of the endoderm cells lining the yolk-sac still remains uncertain. The spread round of the endoderm cells from the periphery of the embryonic endoderm as seen in the macaque yolk-sac, must, in the human, be either very rapid or altogether omitted. If the spreading of endoderm cells round the interior of the exocoelomic

membrane is absent or only partial, then the yolk-sac cells, as distinct from the endoderm cells below the shield ectoderm, must arise by delamination from the precocious mesoderm forming the exo-coelomic membrane as is stated to be the case by Streeter (1937). The absence of a definite double layer of cells over the ab-embryonic pole of the yolk-sac may suggest a migration of endoderm cells from the original embryonic endoderm cells, the migration not having been completed, the single layer present would then be described as mesoderm. But, alternatively, the delamination process may have been delayed over the less well-nourished ab-embryonic region or the second layer of cells may have already disappeared. It is unfortunate that the histological appearances are such that one cannot be certain whether this layer belongs to endoderm or mesoderm.

Whatever the exact mode of origin of the endoderm cells may be, it is evident that this ovum illustrates a stage in the formation of the human definitive yolk-sac intermediate between that described by Hertig & Rock (1941) for ovum No 7700 (12½ days) and that by Brewer (1938) for the 15-day Edwards-Jones-Brewer embryo. Also that this stage, which has not previously been described for man, resembles the similar stage in the 12-somite sloth, and closely resembles the condition postulated by Hamilton, Boyd & Mossman (1945) in their scheme for the development of the yolk-sac.

THE MACAFEE OVUM

The Macafee ovum was recovered from a woman aged 26 years, her last menstruation was from 20 to 26 January, coitus occurred on 22, 25 and 30 January, she was admitted to hospital on 3 February and was curetted on 5 February, the expected date of her next menstruation was 11 February.

The material obtained from the curetting was cut into a diagnostic section and fifty-one serial sections separated from the former by a small interval due to loss of sections. The diagnostic section, of which Pl 2, fig 6 is a photograph, is nearest the centre of the ovum, and shows an endometrium well prepared for implantation. The endometrium is not grossly oedematous and there is no marked leucocytosis and no large haemorrhagic areas. Some capillary sinusoids were found to communicate with the spaces in the outer part of the trophoblast. The glands are dilated, but do not contain blood or pent-up secretion, some of them are being engulfed by the syncytiotrophoblast and their cells are disintegrating in the trophoblast spaces. There is no necrotic zone, and no syncytial masses of peripheral trophoblast were seen.

The ovum, which from the wax-plate reconstruction is seen to be ovoid in form, is very superficially embedded so that a large part of it is exposed and uncovered by uterine tissue. The measurements of the largest section are trophoblast, maximum external 1.13×0.63 mm and trophoblast cavity 0.58×0.33 mm. The trophoblast wall consists of two layers, an inner of large cuboidal cells lining the cavity, and projecting outwards as cell columns, and an outer thin covering layer of syncytium. This syncytium bounds lacunar-like spaces and peripherally forms a continuous layer which constitutes the junctional zone with the maternal stroma. There is a general absence of activity penetrating syncytium from this layer and the line of demarcation between it and the uterine stroma is quite definite. The trophoblast spaces contain

a coagulum and only a small amount of maternal blood cells. The trophoblast is lined internally with a layer of extra-embryonic mesoderm which does not indent the trophoblast columns. In the section nearest the centre of the ovum (Pl 2, fig 6) the mesoderm forms what appears to be an exo-coelomic membrane, but this cannot be traced in the more peripheral sections, there the mesoderm is smaller in amount and forms a much thinner layer. There is no embryonic rudiment.

Discussion

The superficial implantation and the general histological appearance of the specimen indicate that the ovum was probably dying and being cast off by the maternal tissues. The coital and menstrual histories are believed to be reliable, and assuming that the coitus on 25 January was the fertile one, give to the ovum a maximum coital age of $10\frac{1}{2}$ days. The measurements of this ovum, as compared with other pre-villous ova, notably the Hertig & Rock ova (Carnegie Nos 7699, 7700, 7950 and 8020), the Davies-Harding, Barnes, Miller, Dible and West, Kleinhaus, Werner, Scipiadès and Marchetti ova, also place it between 9 and 13 days of age. In appearance it closely resembles that of Carnegie No 7950 (Rock & Hertig, 1942). The engulfment and disintegration of the uterine glands by the syncytium are unusual in an ovum of this early stage of development and indicate that the invasive power of the syncytium must, at one time, have been considerable.

SUMMARY

Two human ova recovered from uterine scrapings are described. The older specimen (14 days) is normal, but is rather superficially embedded. It shows a large yolk-sac with proximal and distal dilatations connected by a narrow tubular duct. The proximal and duct parts are lined with cuboidal cells. The distal expansion is partially lined with flattened endoderm cells and also shows an area one cell in thickness over the ab-embryonic pole. The significance of the appearance of the yolk-sac and the general position of the ovum with regard to other early human ova are discussed.

The younger ovum (10 days) is incomplete. It is superficially embedded, and probably pathological. Erosion and engulfment of the uterine glands have occurred at an earlier stage than is usually described in the human. Its probable age and its general chronological position are discussed.

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EXPLANATION OF PLATES

Figs 1-5 are of the Biggart ovum, fig 6 of the Macafee ovum

PLATE 1

- Fig 1 The ovum *in situ* Sect 81 $\times 38$
 Fig 2 The duct part of the yolk sac The distal end is inferior Sect 77 $\times 375$
 Fig 3 The distal expansion of the yolk sac The end of the duct just appears above, on the left Sect 77 $\times 375$

PLATE 2

- Fig 4 The embryonic rudiment The distal expansion of the yolk sac is not figured Sect 81 $\times 140$
 Fig 5 The opening of the duct part into the distal expansion of the yolk sac which shows its thin embryonic wall below Sect 75 $\times 95$
 Fig 6 The Macafee ovum *in situ* Diagnostic section $\times 65$



1



2

3



OVARIAN AUTOGRAFTS IN MONKEYS

BY ANITA M. MANDL AND S. ZUCKERMAN

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Four main and conflicting conclusions emerge from the extensive literature on the origin and development of ova in vertebrates (Heys, 1931, Everett, 1945). The first is that the parent cells of the ova are segregated very early from the soma, and migrate in man from the yolk-sac (Witschi, 1948) to the genital ridge where they establish themselves in the germinal epithelium. The second combines this view with the assumption that elements of the germinal epithelium derived from the mesothelium of the genital ridges also produce germ cells. The third presupposes an early and extragenital segregation of primordial germ cells, but holds that these all degenerate, and that in the differentiated gonad only the germinal epithelium produces ova. The fourth denies any early segregation of germ cells, and attributes to the germinal epithelium the total potency of the body to produce germ cells.

The unsettled nature of the problem is further exemplified by the literature on the formation of germ cells after puberty. Here there are three main views. The first is that all the ova that are going to appear in the life cycle of an individual do so before puberty. The second is that the germinal epithelium continues to produce germ cells throughout reproductive life, and in phase with the oestrous or menstrual cycle. The third is that not only the germinal epithelium but also other, as yet unidentified, cellular constituents of the ovary produce ova.

Discussion of these various matters has usually followed hard and fast lines, in the sense that most authors have regarded one conclusion about the origin and formation of the germ cells as being incompatible with any other. The evidence also clearly demonstrates the great difficulty of using purely histological observations of normal material as a basis for conclusions about the temporal phases of dynamic processes. Indeed, the issues have become so involved and confused that it would seem unlikely for any of the conflicts to be resolved except by the study of purely experimental material.

One piece of evidence that has been adduced in favour of the view that the germinal epithelium is not essential to the formation of germ cells is the presence of ova in ovaries from which it has disappeared. In an extensive series of experiments on the rat, guinea-pig, cat, and opossum, Moore & Wang (1947) have shown that corpora lutea and active follicles in all stages of development are to be found a year after the germinal epithelium has been eliminated by the application of salicylic acid to the ovarian surface. Follicles in varying stages of development have also been reported in ovarian grafts whose germinal epithelium has been completely replaced by fibrous tissue (e.g., Marshall & Jolly, 1907, 1908, Pettinari, 1928, Breward & Zuckerman, 1949). In so far as it is impossible to differentiate between the persistence of pre-existing follicular elements and the neo-formation of ova from unidentified cells in the ovarian substance, none of this evidence is in itself conclusive, it is however, useful to the solution of the general problem.

It is for that reason that the following data relating to monkey tissue have been assembled in the present paper

The transplantation of ovaries in monkeys was first reported by Marshall & Jolly (1908) These workers grafted the ovaries of an unspecified type of monkey on to the peritoneum of another unspecified monkey At the same time, they removed the host's ovaries and transplanted them also on to the peritoneum When it was killed 2 months later, the host's own ovaries were still in their new position, although they had 'undergone a certain amount of fibrous degeneration', whereas the homo-graft ovaries had been completely absorbed The heterotypic grafting of monkey and ape ovaries into human beings achieved great notoriety in association with the name of Voronoff Hannan (1931) has attempted the reverse procedure and has grafted human ovaries into rhesus monkeys, but without success None of the voluminous literature on ovarian grafts in Primates contains any quantitative appreciation of the results or of the histological changes

MATERIAL AND METHODS

The tissues whose histological study forms the basis of the present report were taken from two adult rhesus monkeys (*Macacus mulatta*) which were used in experiments (in 1933 and 1934) designed to disclose a possible neural factor in the control of menstrual bleeding (Zuckerman, 1938) An account of the experiments has not been published hitherto, and is therefore given here

Experiment 1 (Mm 77)

The animal, which weighed 5.3 kg, was operated upon on the first day of a menstrual cycle After being anaesthetized with nembutal, given intraperitoneally, the abdomen was opened, and the ovaries removed A number of small follicles, none bigger than 2 mm were visible on the surface of the right ovary, and on its supero-medial aspect was an old corpus luteum, with a surface scar The left ovary showed the stigma of an apparently very recent corpus luteum The ovaries were transplanted into the eyes The lids were retracted at the lateral canthus, and after trimming at the hulum and both poles, the ovaries were transplanted whole into the posterior chambers of the eyes There was some escape of vitreous humour, but very little bleeding Tenon's capsule but not the sclera was stitched

The animal rapidly recovered from the operation and retained effective vision She died 10 months after the transplantation

The eyes were fixed in formalin, serially sectioned at 20μ and stained with Meyer's haemalum and eosin

In the 10 months after the first operation, a study was made of the effects of interruption of sympathetic impulses along the cervical sympathetic chains and spinal cord Uterine bleeding occurred for the first time 158 days after the transplantation of the ovaries The animal then experienced three menstrual cycles lasting 44, 45 and 42 days respectively She died on the sixteenth day of the succeeding cycle, her weight at death being 5.1 kg

Histological study

Every section was examined, and all follicles the ova of which showed a nucleolus were counted. They were classified into six types (see below), and differentiated as normal or atretic. This classification is similar to the one previously used by Green & Zuckerman (1947).

Experiment II (Mm 88)

This adolescent rhesus monkey weighed 4 kg when operated on. The transplantation was carried out in the same way as in the first experiment, except that the sclera as well as Tenon's capsule was sutured. She made an uneventful recovery, but her vision was much impaired by the operation.

The animal died 11 months later. During this interval she was given four courses of gonadotrophin injections, and a study was made of the effects on the menstrual cycle of general vaso-motor shock and of interruption of sympathetic impulses in the cervical chains.

Four phases of uterine bleeding occurred during the 11 months after the ovaries were transplanted.

At autopsy no ovarian tissue was found in relation to the Fallopian tubes of either animal.

RESULTS

Mm 77

Left ovary The ovary had fixed itself to the posterior surface of the lens, its lateral extremity on the attachment of the suspensory ligament. The retina in many places showed pathological change.

The maximum dimensions of defined ovarian tissue are 7.5 mm long, 2.65 mm wide and 1.5 mm thick. The tissue is well vascularized and compact, and is covered behind by flattened cells different in appearance from normal germinal epithelium. No epithelium covers the anterior surface, which is attached to the capsule of the lens.

The ovarian stroma contains many epithelial elements. Healthy ova are absent, but there are the remains of a few primordial follicles undergoing atresia. There are many follicular cysts, the largest of which is irregular in shape and 0.7 mm in diameter. A large corpus luteum is present.

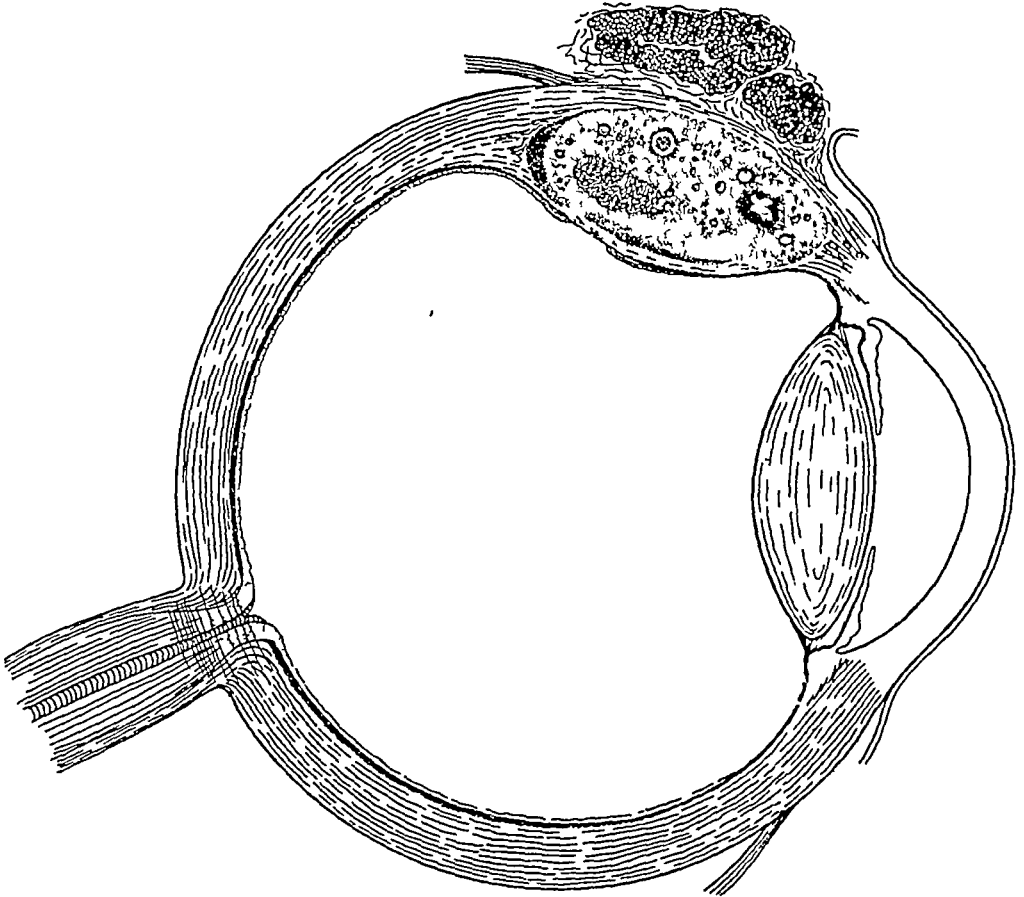
Right ovary The ovary implanted itself into the lateral part of the eyeball within the sclera, its anterior pole abutting on the lachrymal gland. The ovary is compact and had retained a more normal ovarian appearance than its fellow (Text-fig 1). Its maximum dimensions were $8.8 \times 5 \times 3$ mm.

The retina of this eye appeared to be healthy, and the lens was not occluded.

The ovary contained three distinct, and two ill-defined corpora lutea, as well as many follicles (Pl 1, fig 1). The largest follicle measures 2.5×2.2 mm in the stained preparations. The two most recent corpora lutea (Pl 1, fig 2) are situated in the half of the ovary adjacent to the lachrymal gland, and are separated from each other by a large atretic follicle. There is no sign of luteinization in any of the larger follicles, nor of any germinal epithelium.

The follicles were classified as follows

- Type 1 single layer of flattened or rounded granulosa cells
- Type 2 single layer of cuboidal granulosa cells
- Type 3 two layers of cuboidal granulosa cells
- Type 4 three layers of cuboidal granulosa cells
- Type 5 more than three layers, but no antrum
- Type 6 antrum present



Text fig 1 Schematic diagram of right ovary of Mm 77

The total number in the ovary were

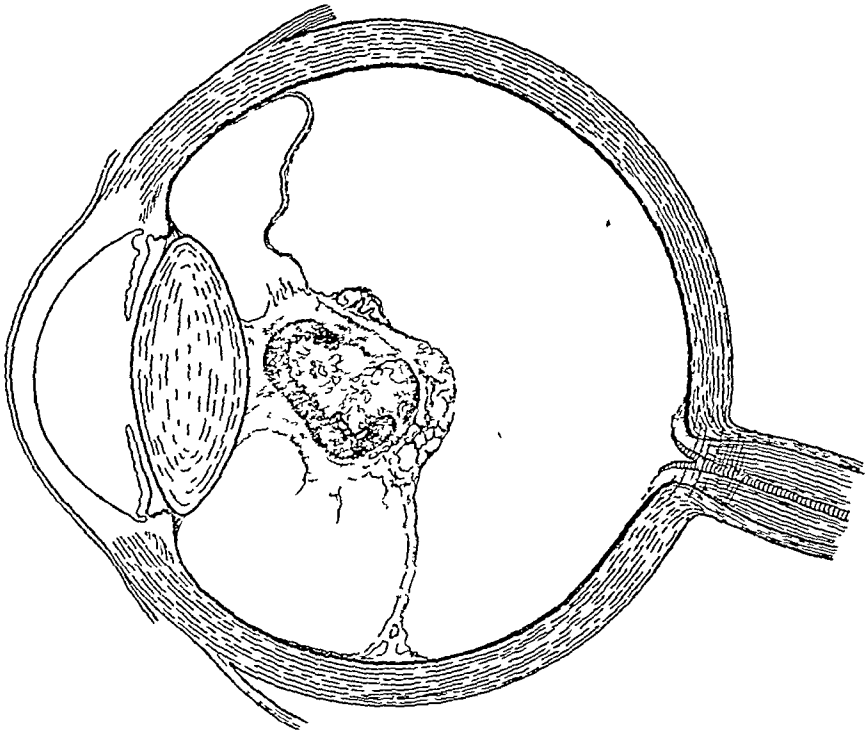
	Type 1	Type 2	Type 3	Type 4	Type 5	Type 6	Total
Normal	121	6	6	2	4	—	139
Atretic	145	29	24	6	7	1	212
Total	266	35	30	8	11	1	351

Mm 88

Left ovary The ovary had implanted itself into the base of the ciliary body, which was completely disorganized. It measured $2.5 \times 2.4 \times 1$ mm, and was not as compact as its fellow. In places the cortical zone appears to be incomplete. There was no sign of germinal epithelium.

No healthy follicles containing ova were found. There were many degenerating follicles enclosing hyaline plaques, whose indistinct outlines were reminiscent of a degenerating zona pellucida. Some follicles had a vascular core, others seemed to be solid balls or cylinders of granulosa cells.

Right ovary The ovary has a typical ovoid form and except at one point is embedded completely in a mass of new tissue which stretches obliquely between the lens in front and the posterior wall of the vitreous behind (Text-fig. 2). The choroidal



Text fig. 2 Schematic diagram of right ovary of Mm 88

attachment of this mass of tissue is in the form of a prismatic pedicle. The maximum dimensions of the ovary in the stained preparations are $3.75 \times 2 \times 1.8$ mm. It consists of a core of vessels and fibrotic atretic follicles, which have undergone hyaline change, surrounded by dense chromophil cortex consisting of closely packed stromal cells and ovarian epithelial elements. The maximum diameter of the cortical zone in the stained preparation is about 0.2 mm. Germinal epithelium was not recognizable.

Within the dense ovarian cortex can be recognized aggregates of granulosa cells and cornified plaques of the kind observed in the left ovary. There is only one large follicular cyst, irregular in shape, 0.6 mm in diameter. No healthy or atretic follicles with nucleus and nucleolus at any stage of development could be seen, and the appearances suggested a greatly enhanced rate of follicular atresia.

Uterine bleeding occurred in this animal, in spite of the absence of all cyclic

structures in the ovary This observation can be correlated with the fact that oestrous cycles continue in mice whose ovaries after X-ray sterilization no longer exhibit any cyclic changes (Parkes, 1926, 1927 *a*, *b*, and *c*)

DISCUSSION

The view generally held by those who attribute the development of ova entirely to the germinal epithelium is that the germ cells have a short life cycle which is correlated with the length of the oestrous or menstrual cycle (Allen, 1923, Evans & Swercy, 1931) The very high rate of atresia that is always to be observed in the ovaries of mammals undoubtedly supports this view, and makes it difficult to suppose that follicular elements observed in an ovary a year or so after the disappearance of the germinal epithelium can represent the survival of pre-existing ova

Unless ova have a long and not a short life cycle, it is impossible not to accept Moore & Wang's (1947) view, based as it is on observations of the ovaries of the rat, guinea-pig, cat and opossum, a year after the germinal epithelium had been destroyed with salicylic acid, that the presence of an active germinal epithelium is not essential for oogenesis This view obtains some support from the tissue-culture experiments of Martinovitch (1938) These showed that oocytes are formed after most of the germinal epithelium cells have emigrated into the culture medium As the cultures survived for only 1 month, however, it may be, as Martinovitch himself suggests, that this evidence should not be taken to indicate more than that the germinal epithelium is not essential to the growth of the ovum after synapsis

Further support for the view that the germinal epithelium is not essential is, however, afforded by observations which suggest that ovarian tissue can regenerate in mice after complete removal of the ovaries (e.g. Parkes, Fielding & Brambell, 1927) On the other hand, the work of Heys (1931) and Pincus (1936), which suggests that 'complete removal' in a mature rat or mouse is almost impossible because of the folding and lobulation of the ovary, casts some doubt on this line of evidence The conclusion is, however, also supported by the fact that ova are not formed in a mouse ovary after sterilization with X-rays, in spite of the persistence of an apparently normal germinal epithelium (Parkes, 1926, 1927 *a*, *b*, and *c*, Brambell & Parkes, 1927, Brambell, Parkes & Fielding, 1927 *a*, *b*, Parkes, Fielding & Brambell, 1927) Here, on the other hand, it can be argued (Pincus, 1936) that whatever its appearance, the epithelium is, in fact, abnormal, in so far as X-rays have suppressed its capacity for oogenesis The evidence also lends itself to a third interpretation Everett (1943), whose observations suggest that the 'somatic epithelial tissue' of the germinal epithelium is distinct from its germ-cell producing elements, considers that such X-ray experiments merely show that the 'somatic epithelial tissue'—the surface germinal epithelium which never produces ova—is not damaged

The fact that grafted ovarian tissue may contain both follicles in active development and luteal tissue long after it is implanted may also be taken as an indication that the germinal epithelium is not essential for oogenesis This line of evidence, however, hinges on two sets of considerations—the clear demonstration that germinal epithelium is no longer present in the graft, and the speed at which it disappears after grafting

Some writers, e.g. Pincus (1936), hold that the germinal epithelium of grafts does not normally disappear, while others, e.g., Herlitzka (1900) and Marshall & Jolly (1907, 1908), state that it usually disappears entirely. The latter authors have reported normal ovarian tissue, presumably without germinal epithelium, in an autograft in a rat after 14½ months. The survival of the germinal epithelium, however, clearly varies with circumstances. Pettinari (1928) suggests that the cells are destroyed when they become adherent to any other tissue. Everett's (1943) experiments seem to confirm this view. When he transplanted ovaries within their bursa on to the kidneys of mice they frequently took without fusion, and oogenesis continued. When they were transplanted without the bursa, fusion occurred, the germinal epithelium disappeared, and oogenesis ceased. In the experiments described in the present paper, and also in those of Breward & Zuckerman (1949), the germinal epithelium was undoubtedly replaced by other cellular elements—especially where it was adherent to another tissue. On the other hand, in these experiments oogenesis, as manifested by the presence of young follicles, had not stopped.

The speed with which the germinal epithelium of an ovarian graft changes has been remarked upon by several authors. According to Athias (1920) and Pettinari (1928) swelling and hyperplasia occur within 2 or 3 days, and are associated with many signs of degeneration. Athias found cords of germinal epithelium growing into the cortical zone of the graft, and he suggests that ova are formed from these downgrowths. Pettinari, however, who has observed the same change, regards it as a reaction to irritation and not an oogenetic phenomenon. Tamura (1927) has addressed himself closely to the question of the survival of the germinal epithelium, and has made an extensive series of ovarian grafts onto the kidneys of male mice, the tissue being placed just within the kidney capsule. His observations were limited to the first 35 days after grafting. He found that in general the follicles existing in a graft at operation disappear, and that they are replaced by the germinal epithelium which remains functional and normal in appearance. He makes the interesting observation, however, that where adhesion occurs, the germinal epithelium disappears and he therefore concludes that 'if the germinal epithelium is unimpaired it proliferates'. Hannan (1929), however, reports the very rapid disappearance of the germinal epithelium of ovarian homografts in the deep fascia on the backs of rabbits. Butcher (1932) also refers to the disappearance of germinal epithelium in some ovarian autografts in the kidney fat of rats. In others he describes the persistence of the epithelium. His work, like Tamura's, also suggests that the germinal epithelium is in general more resistant to impaired vascular conditions than are the existing ova and follicles, which it replaces by proliferation.

It is obvious that the fate of the germinal epithelium in ovarian grafts is very variable. It is equally clear that it is almost impossible to be sure that an epithelium that has apparently been replaced by connective tissue does not persist functionally either within that tissue or as ingrowths into the substance of the ovarian tissue. Desai (1942) has claimed that 'pre ova' can be recognized within the ovarian stroma. Derivatives of the germinal epithelium which are not definitive ova may very well be present as 'pre-ova' within the substance of an ovarian graft.

It would also seem that more has been made than the evidence warrants of the 'disappearance of germ cells in ageing grafted ovarian tissue'. The finding has

usually been taken to support the view that the germinal epithelium is essential to oogenesis. In the observations reported in the present paper the number of ova per grafted ovary was not one-hundredth of the normal. Corresponding observations have been made by other workers. Nevertheless, it would be quite unjustifiable to assume, even though it may be the correct explanation, that this is so because no germinal epithelium was present, or that the same picture would have been seen if the tissues had been studied a year later than they were. Before reaching so final a conclusion, it would be necessary to carry out a series of quantitative experiments in which different-sized ovarian fragments were implanted into different sites, and examined at considerably longer intervals after grafting than has been the rule. Only if it were shown that there is in general a time-correlation in the disappearance of ova from grafts whose germinal epithelium had been lost could it be argued that in these circumstances ovarian stroma is unable to replace germinal elements.

A new factor has recently been introduced into the problem by the emphasis which Dornfeld and his colleagues lay on the differential proliferative activity of the area of germinal epithelium to which the suspensory ligament of the ovary is attached (Slater & Dornfeld, 1945, Vincent & Dornfeld, 1948). According to these workers, this represents almost the only site at which the germinal epithelium of the rat shows proliferative activity—at any rate up to the sixtieth day of life. Their view is that the associated parts of the oviduct and fimbria, through the ribonucleic acid they produce, exercise a stimulating and evocative effect on the adjacent area of the germinal epithelium, which at the hilum forms a solid block of cells in the substance of the ovary. Several points need to be settled before this hypothesis can be related to our knowledge of the behaviour of ovarian grafts. The existence of an area of hyper-activity in the germinal epithelium of mature animals of several species has first to be established. Its dependence on an induction stimulus from the oviduct also needs to be established. Even if oogenesis in grafts lacking a germinal epithelium can be related to an ingrowth of germinal epithelium similar to the one which Dornfeld and his colleagues have shown in the rat, it is clear, in experiments of the kind reported in the present paper, that it cannot be attributed to the local diffusion of a chemical stimulus from the oviduct.

SUMMARY

1 The ovaries of two rhesus monkeys (*Macacus mulatta*) were transplanted as whole autografts into the eyeballs. In the first experiment (Mm 77) they became established in the vitreous of one side, and in the sclera of the other. In the second (Mm 88) they became fixed in the vitreous on both sides. The first animal lived 10 months and the second 11 months after the transplantation.

2 Cyclical uterine bleeding occurred in both animals after the transplantation.

3 In the first animal one ovary contained a number of small follicles undergoing atresia, and a few cystic follicles. The other ovary, which had embedded itself in the sclera, contained many healthy follicles and also three distinct and two ill-defined corpora lutea.

4 In the second experiment, in which both ovaries had become fixed in the posterior chamber, neither contained any healthy follicles or corpora lutea. Soon after their appearance ova underwent atresia.

5 Germinal epithelium was present in only one of the four ovaries. None was present in the ovary which had embedded itself in the sclera, and which was the most active of the four.

6 These observations are discussed in relation to general views about the part played by the germinal epithelium in oogenesis.

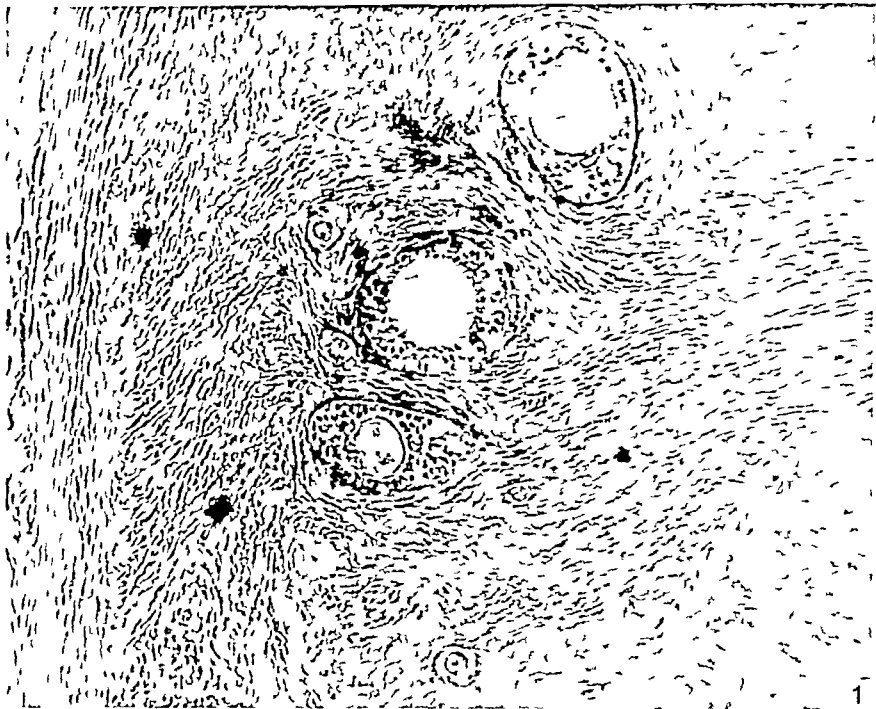
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EXPLANATION OF PLATE

- Fig 1 Young healthy follicles in peripheral part of right ovary of Mm 77, showing the absence of germinal epithelium $\times 120$
- Fig 2 Corpus luteum in right ovary of Mm 77 $\times 50$



STUDIES ON HAIR GROWTH IN THE RAT

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In seeking to answer such questions as, what makes a hair start growing or cease growing, it is necessary to increase our knowledge of the structure of hair follicles and their immediate environment, and particularly their relation to the vascular system. The first part of this paper is concerned with these structural aspects, while the later sections deal with the experimental investigation of the transmission of growth from regions of activity into regions where the hair follicles are quiescent.

The mode of hair replacement varies in different animals and has a constancy in relation to species. Three principal types of follicle activity determine the various patterns:

- (1) Continuously active as in wool and human head hair
- (2) Periods of activity and inactivity with neighbouring follicles in different phases
- (3) Periodic activity with neighbouring follicles in the same phase

The third type is characteristic of the adult rat where active growth is confined to a relatively narrow zone, commencing ventrally and spreading dorsally over the trunk so that at a given time the major portion of the skin is non-active. In the head region and around the limbs the pattern is more complex, but the same essential feature is invariable, viz. the periodic succession of growth waves and intervals of quiescence. Some details of the growth pattern for the rat have been given by Dry (1928), Butcher (1934) and Haddow, Elson, Roe, Rudall & Timmis (1945). The position of the wave may be demonstrated very easily in the living animal by clipping the hair over one side and then observing in the following few days the band along which rapid hair growth appears. Another method which is very useful in the case of white rats is to dye the coat with proflavine and then observe the position of the growth wave by the appearance of non-dyed new hair which stands out in sharp contrast to the dyed hair of the quiescent areas (Pl 1, fig 1, Pl 4, fig 10). In the present work the method of clipping was used in most cases.

The regularity of the growth pattern is quite characteristic in post-pubertal animals of both sexes, but there is some disturbance of the pattern in old age, during lactation, and in states of nutritional deficiency and disease. Our main observations have, therefore, been made in non-pregnant, pre-senile animals so as to avoid complicating factors. Stock albino rats were used throughout, except that in the denervation experiments hooded and black rats were used as well as albinos.

HISTOLOGY

Microscopic examination of skin sections in the region of a laterally placed wave shows that structural changes are present in a sharply defined zone that corresponds in position with the observed wave of hair growth in the living animal (Pl 2, figs 2 and 3). The inactive hair follicles lie quite high in the dermis and do not extend down

There was no evidence that the vessels supplying the capillaries associated with the hairs were isolated from other cutaneous systems. There is, probably, continuity between vessels of the superficial and the deep layers, including those in the panniculus. When mammary gland tissue is present in sections of injected rat material it is seen to be richly supplied with capillaries (Pl 4, fig 9). During lactation in the rat there is some interference with the normal pattern of hair growth, while reduction of hair growth during lactation has been recorded in experiments with guinea-pigs and sheep (Strangeways, 1933, Bosman, 1935). If the activity of the mammary gland in any way diverts the blood from other parts of the cutaneous field, an alteration in hair growth would not be surprising in view of the evident association between hair growth and vascularity. Further, it has been reported (Mottram, 1945) that in mice, which also present a periodic hair-growth pattern, epidermal warts and the epidermis show reduced growth during the passage of the wave, and resume active growth after the wave has moved on, here again one can see a possible connexion between the observed phenomenon and the condition of the vascular bed.

EXPERIMENTAL OBSERVATIONS

Various experiments were devised in order to examine the factors which influence the waves of hair growth.

A Effect of denervation

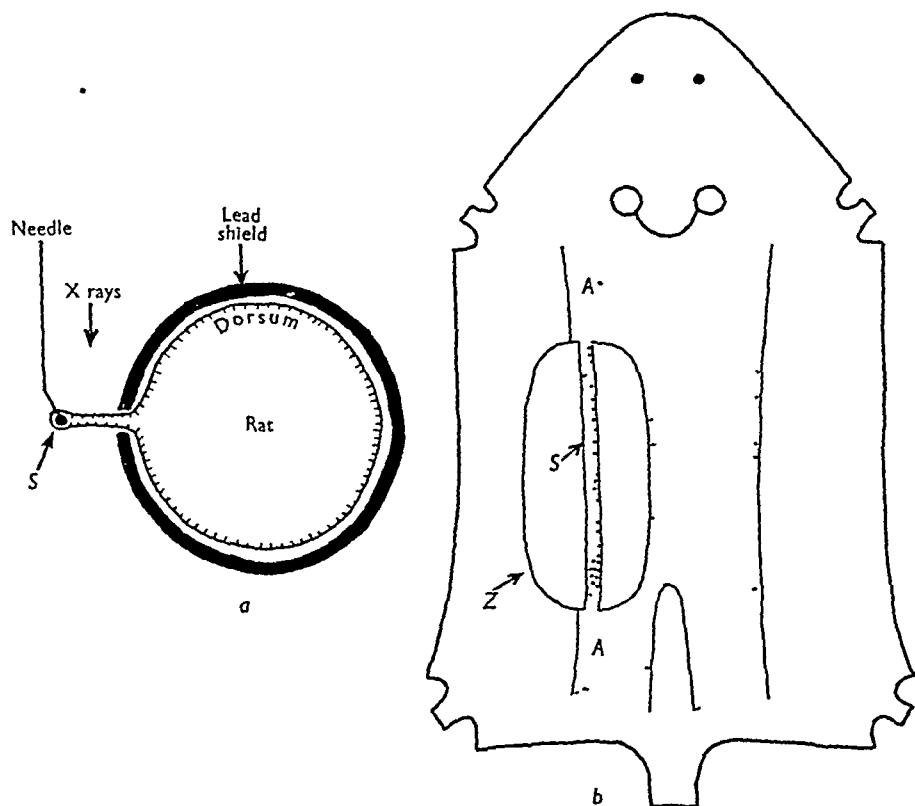
A longitudinal incision was made on one side of the body parallel to the mid-dorsal line from the level of the shoulder to the hip, and the skin retracted sufficiently to allow exposure of the segmental nerves. These were picked up with a fine curved needle and severed so that the major portion of the body wall from shoulder to hip was denervated, the only nerves remaining intact being those derived from the posterior primary divisions and not concerned with skin innervation except in the dorsal skin medial to the incision. The nerves to the panniculus carnosus were severed at the rostral end of the incision. They were easily located deep to the panniculus as a leash of fine branches and, although there is no evidence that they are cutaneous in the usual sense, their severance removed any possibility of their playing a part in the subsequent hair-growth pattern. The incision was closed by sutures, and the wound allowed to heal prior to making any observation of the wave. These animals were observed for periods of 3-5 months following the operation and no alteration in the pattern of growth was detected. Occasionally there was a suggestion of rather less vigorous growth of hair on the operated side, but in all cases the growth wave retained its normal pattern of ventrodorsal progression and the symmetry of the process on the two sides was retained.

B Effect of exposure to X-rays

An anaesthetized animal was enclosed in a lead-protecting shield, along the side of which a narrow slot had been cut. A longitudinal fold of skin was withdrawn through the slot and exposed to a dose of X-rays (3000 r)*. A steel needle, inserted subcutaneously, supported the crest of the fold and thus cast a shadow on the skin

* Dose, 3000 r, dose rate, 315 r/min, radiation, 80 kV, half value layer, 1.3 mm Al

(Text-fig 1a) The result was an epilated zone of skin approximately 4 cm long and 2 cm broad, with a narrow non-epilated strip *S* running along the middle of the zone (Text-fig 1b) During subsequent observations over a period of 8 months, a very scanty growth of hair was observed on the irradiated area and it was assumed that the activity of most of the follicles had been stopped. Observation of growth on the two sides of the animal showed that the wave was not altered by the presence of the large epilated area. The wave passed towards the dorsal line in front of, behind and



Text fig 1 a Method adopted to irradiate an isolated area of skin. Needle prevents irradiation of skin at *S*. b Diagram of rat pelt to show zone of epilation *Z* on left side. *A* active hair growth. *S*, strip of non irradiated skin running through epilated zone *Z*.

above the epilated area, symmetrically with the wave on the opposite side. Within the irradiated area the narrow strip of non-irradiated skin, *S*, came into activity simultaneously with *A* at the same level on the main region of the body. A correspondence between growth at *S* and *A* occurred during each of the four cycles of growth observed.

It must be pointed out that the degree of epilation, though extensive, was not complete. Choosing the most suitable dosage creates problems in itself. Observations made during the course of the present experiments suggest that the necessary epilation dosage depends on the phase of the hair growth at the time of irradiation.

C Effect of skin excision

Strips of skin nearly 1 cm wide (down to the hypodermis but not including the panniculus carnosus) were removed from the left side in two animals. These strips extended forwards from the hip half-way towards the shoulder in one animal and rather farther in the other. The upper and lower edges of the resulting gap were approximated by sutures and the wound allowed to heal. In the first cycle of hair growth, which followed quickly after the operation, growth proceeded to the scar on the operated side, paused a few days and then proceeded beyond the scar to the mid-line. This revealed little except that the scar line was a region of growth discontinuity. In the second cycle after the operation, growth proceeded to the mid-line in front of the level of the scar on both sides. More posteriorly, growth extended to the mid-line on the non-operated side, but on the operated side it extended during the same period only to the level of the scar. Growth eventually proceeded above the level of the scar after a pause of approximately 2 weeks. One animal survived to a third cycle, and growth beyond the scar on the operated side was again much delayed.

The significant feature of this experiment is that the symmetry of the growth waves on the two sides is affected by removal of a longitudinal strip of skin. Growth is delayed dorsalward of the site of skin removal, either in consequence of removing the skin, or because of the scar which inevitably forms at that level.

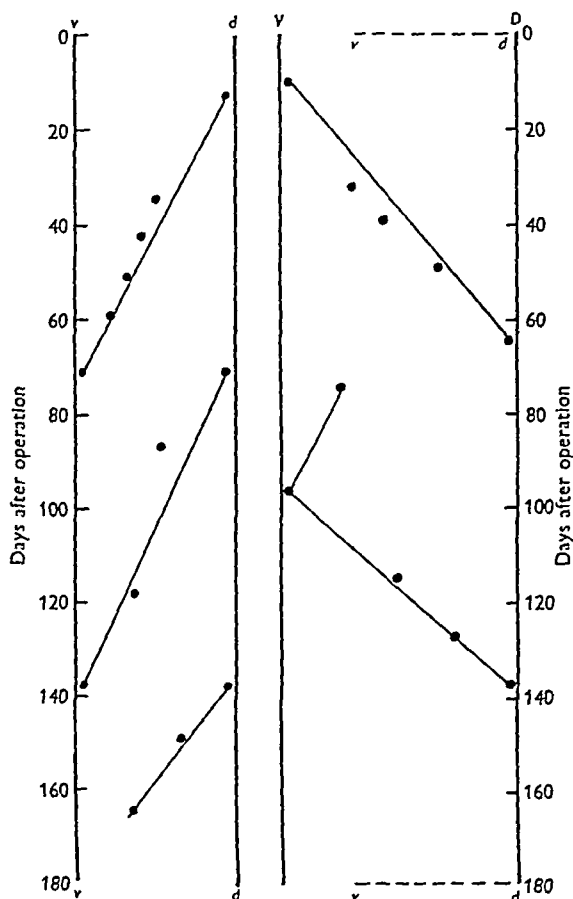
D Effect of skin rotation

The purpose of the experiments described under this heading was to place side by side areas of skin which were in quite different phases of growth. Observations could then be made as to whether the phases altered so as to become the same, or whether they remained as different as before the operation. A number of young post-pubertal rats was treated in the following way.

A square of skin (3×3 cm) was outlined on one side by incisions extending down to, but not including, the panniculus carnosus, the edges of this square were freed, but the main area of the square was not detached from its subcutaneous connexions so as to leave an intact blood supply. The square was then rotated through 180° and sutured in position. This meant that the original ventral margin became dorsal and the caudal margin became rostral. Healing occurred readily, and in 10 days the site of operation was clearly defined by the reversed hair slope on the square of rotated skin (see Trotter & Dawson, 1931, for similar result in guinea-pig). Observations over a period of several months were then made on the growth waves of the hair. It was found that within the rotated square the growth wave was reversed and passed from the dorsal to the ventral margin. To demonstrate this effect an animal was subjected to the above operation and after healing of the wound the whole fur was dyed with proflavine. Two weeks later, it was noted that the fur was growing white on the upper third of the rotated area, while on the body surface outside this area the new white hair was coming in along a ventrolateral zone. Ten days later the animal was photographed in colour, and a black and white reproduction of this is given in Pl 4, fig 10. Within the rotated area growth has proceeded downwards from the upper edge, while on the rest of the body, growth of hair has proceeded

upwards as far as the mid-lateral line. As usual, at the lines of incision there is some distortion of hair slope, this may be noted along the caudal incision in Pl 4, fig 10

The chart shown in Text-fig 2 was constructed from an extended series of observations on another animal and shows the progress of the growth wave from dorsal to ventral regions within the rotated area of skin $d-v$. All the experimental



Text-fig 2 Chart to illustrate progress of successive hair growth waves on rotated skin, $d-v$ in comparison with waves on main area of body, $I-D$. The position of the rotated area on the body surface with reference to the mid dorsal line (D) and mid ventral line (V) is indicated by the interrupted line v d

animals tended to conform to this plan. In none of the animals did the movement of growth on the rotated skin proceed in other than a dorsoventral direction, nor was there any suggestion of interference between the ventrodorsal growth on the main areas and the oppositely directed growth on the rotated skin. The general conclusion is that the wave of growth on the rotated skin proceeds independently of that on the main areas, but tends to travel more slowly. In some cases this retardation was very marked.

DISCUSSION

The results of the experiments involving section of nerves to the body wall suggest that the pattern of hair-growth waves in the rat is independent of the innervation of the skin in the regions concerned. It can be assumed that the cutting of the segmental nerves involved not only somatic fibres, but also, the sympathetic post-ganglionic fibres travelling in the nerves to the vessels of the body wall including the skin. The vascular pattern associated with the persisting growth wave could not therefore be explained on the grounds of any nervously controlled vasodilatation.

The increased vascularity within the growth wave is largely due to new capillary networks in the dermis and hypodermis. The most extensive capillary network is found around the lower half or third of the follicle. This external supply to the follicle wall is of far greater extent than the internal supply to the follicle papilla, and it is possible that the external root sheath reacts with this capillary system, adding to or removing substances from the blood before this passes through the papillary vessels. In the smaller hair follicles the circulation about the follicle wall is very much less extensive than that shown at C and E (Pl. 3, fig. 7). The follicles which produce the largest overhairs, notably monotrichs, have the most extensive capillary system. These fibre types contain a considerable bulk of keratin, as can be seen in fig. 7 of the paper by Haddow & Rudall (1945), and a most reasonable explanation of the extensive circulation to the follicle wall would be that it is associated with the synthesis of intracellular protein in the hair cuticle and cortex.

In our studies of the follicle circulation we have not been able to observe the capillaries to the papilla except in a few isolated cases. According to Johnson, Butcher & Bevelander (1945) the papilla markedly alters its shape during the catagen phase (Dry, 1926), becoming narrow and elongated. There may well be changes in the circulation to the papilla at this time, though the circulation to the follicle wall does not appear to change. It should be pointed out that the distribution of capillary vessels corresponds approximately with that of the supposed phosphatase activity in the papilla and the follicle wall. As the capillaries themselves show abundant phosphatase activity it is not certain to what extent the recorded distribution of the enzyme is due to a blurred distribution of capillaries. On such a view the reduced phosphatase activity of the papilla at the catagen phase may represent the reduction or degeneration of its capillaries. But if we accept the recorded distribution of alkaline phosphatase as being substantially correct and not due to capillary phosphatase, then the following valuable correlations may be made: the development of this phosphatase activity is associated with the development of a rich network of capillaries, and the disappearance of phosphatase is associated with the disappearance of the special capillary system.

In the initiation of growth at the wave-front it can be debated whether the follicle activity or the increased vascularity has priority. The periodic nature of hair growth would seem to be due to a fixed genetic pattern. Having entered the resting stage, no proximity to an active circulation will cause growth to start again until the required resting stage has been completed. Thus a wave-front moves forward and establishes a new front on meeting follicles which have not rested sufficiently long to recommence activity. It has been shown that there are special capillary branches

to follicles which are in the completely quiescent stage (Pl 3, fig 7A), and such follicles may be affected via their residual capillary system by the presence of adjacent highly vascular areas of the type illustrated at B-E. In other words, the presence of an increased vascular system at the wave-front may help to limit the resting period in contiguous areas so that these are generally the next regions to show follicle activity.

The experiments which have been described, involving X-ray epilation, skin excision and skin rotation, were performed with the object of studying the progress of the growth wave in an area isolated in some way from its normal contacts with undisturbed skin. Isolation was achieved in one set of experiments by X-ray epilation and in these cases the possible influence of skin incisions was avoided, the excision of strips of skin or the rotation of an area of skin allowed the juxtaposition of regions in different phases of activity, in the one case by removal of the intermediate phases and in the other by the mere rotation.

The particular form of the epilated area resulting from the X-ray irradiation left an isolated strip of non-irradiated skin (Text-fig 1). During four cycles of hair growth the narrow non-irradiated strip passed into the active phase at the same time as the non-irradiated areas at the same level elsewhere on the body. There was no evidence that growth on the strip *S* (Text-fig 1) was in any way delayed because of absence of contact at its ventral edge with a full complement of actively growing follicles. By contrast, where a strip of skin was excised along one side of the body there was a definite delay in the ventrodorsal passage of the growth wave. This is the only example we have at present where conditions have been imposed which produce a barrier to the transmission of the wave and we do not yet know whether this barrier is due to scar formation or to the removal of necessary intermediate phases.

From the skin-rotation experiments we have concluded that the direction of the wave is inherent in the skin, the wave of growth proceeds independently within the rotated area as if the latter were isolated from surrounding skin. Just the opposite conclusions were reached by Butcher (1936) who states 'the time of hair growth is dependent on factors resident within the animal and not in the skin'. The principal differences between our approach and Butcher's were as follows: (1) We have dealt exclusively with adult rats, whereas his experiments were performed on prepubertal material. (2) We studied areas of skin of much greater dimensions than he did. (3) The skin in our rotation experiments was never completely detached from the underlying tissues, and so the blood supply remained relatively intact. These points are sufficient to account for the different findings. From the observations of Heringa & Weidinger (1942) it is reasonable to assume that in prepubertal animals the skin is gradually becoming more fibrous and less highly hydrated. It is also reasonable to suppose that the rate of diffusion of activating substances through the skin decreases with increasing age. On this basis we may be able to account for the fact that the hair-growth waves change from the broad prepubertal type described by Butcher (1934) to the narrow type illustrated in Pl 1, fig 1. It is also probable that the structure of the scar tissue will be much more densely fibrous in older animals, and this could account for the 'barrier' effect resulting from excision of strips of skin and for the apparent isolation of rotated areas of skin.

The less rapid growth which we observed on the rotated skin might conceivably be attributed to a varying degree of interference with the normal circulation. In Butcher's experiments, which involved a complete severance of all vascular connections, he found that the follicles retracted towards the epidermis in the donor skin without completing the normal cycle of growth. The normal resting period of the follicle had not been established and thus its inertia to regrowth was not present. It is therefore understandable that the oncoming vascular wave should stimulate the donor skin to active hair growth. Where the normal cycle of follicle activity was maintained we failed to detect any stimulating effect of one area on another when hitherto unrelated edges were brought into contact by skin rotation. Butcher's experiments indicate, however, that such an effect does in fact exist. Our experiments show that this effect, if present, is small compared with the inertia of the resting period or is decreased in adult animals by the scar tissue.

It seems reasonable to conclude that the progress of the hair-growth wave is due to interaction between the resting-stage inertia and the stimulus of neighbouring vascular activity.

SUMMARY

1 The histological features of the skin and the hypodermis in the rat are compared in areas showing active hair growth and in quiescent areas.

2 The density of the capillary bed about the hair follicles varies according to the stage of the hair-growth cycle. The distribution of capillaries is similar to that of alkaline phosphatase activity.

3 Principal differences between the circulation to hair follicles in the rat and the sheep are described.

4 Denervation of an area of skin has no effect on the passage of the wave of hair growth over it.

5 Various experiments, involving skin excision, skin rotation and X-ray epilation, were performed in order to study the processes involved in the transmission of hair-growth waves from venter to dorsum and we have concluded that this is due to interaction between resting-stage inertia and the stimulus of neighbouring vascular activity.

We are indebted to Dr F W Spiers, Lecturer in Medical Physics in the University of Leeds, for his advice concerning the irradiation of the skin and for providing the facilities for this aspect of the investigation. To Mr W Hutchinson of the Anatomy Department we are grateful for his assistance at operations and for the preparation of the histological material.

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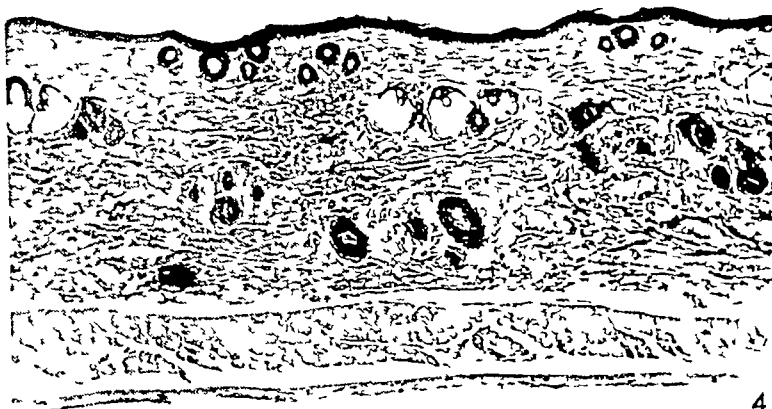




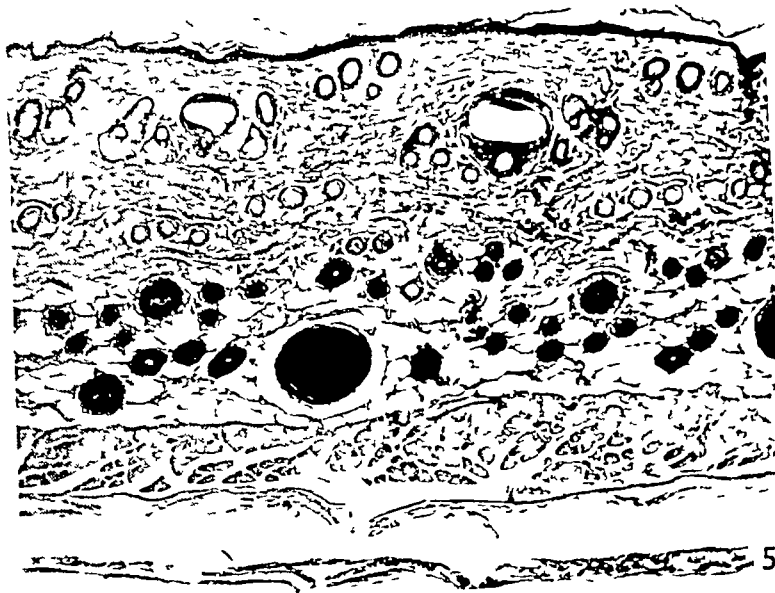
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DURWARD AND RUDALI — STUDIES ON HAIR GROWTH IN THE RAT

INTERNODE LENGTHS IN THE NERVES OF FISHES

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INTRODUCTION

Studies on mammals and amphibians have begun to reveal the principles that govern the spacing of the nodes of Ranvier. These gaps in the myelin are normally placed farther apart in larger than in smaller fibres (Ranvier, 1875, and others, see discussion later), but after regeneration in the adult this relationship no longer obtains, all internodes are then short (Young, 1945*b*, Sanders & Whitteridge, 1946, Hiscoc, 1947, Vizoso & Young, 1948). This suggests a connexion between growth and internode length, which is confirmed by the finding that the line relating internode length to diameter becomes progressively steeper during development (Young, 1945*b*, Hiscoc, 1947, Vizoso & Young, 1948). Further, in the legs of amphibians the internodes are longest where the amount of growth is greatest (Takahashi, 1908, Hatai, 1910).

The few studies made on fishes (Ranvier, 1872, Key & Retzius, 1876) show that these animals possess internodes several times longer than any reported from mammals or amphibians. We have therefore studied the internode length in nerves of fishes of various lengths, and also in nerves taken from parts of the body likely to have differing growth rates. In particular we have been interested in long-bodied fishes, such as the eels, where the nerves increase several times in length during the period after medullation.

METHODS

The nerves, with the exception of those from *Torpedo*, were all obtained at Plymouth during the summer of 1947 from the elasmobranchs *Raja clavata*, *Scyliorhinus canicula* and *Torpedo ocellata*, and the teleostean *Conger conger*. The nerves used were mostly the lateral line and branchial branches of the vagus. After dissection, the stretch of nerve to be taken was measured while still in position, then cut with scissors and attached to a piece of slotted cardboard at its original length. It was fixed in 10 parts of 40 % formaldehyde diluted with 45 parts of sea water and 45 parts of distilled water. The salts in such a solution will be approximately isotonic with those in the blood (Young, 1933). The nerves were stored in this medium until required some months later. They were then washed, and stained with a 1 % solution of osmium tetroxide for 24 hr. After further washing in distilled water they were passed to a mixture of glycerin and water in the proportion of 2 to 1 for 3 days to facilitate teasing.

Single fibres were obtained by dissociation in pure glycerin under a dissecting microscope with fine mounted needles, care being taken to minimize damage or stretching. The internodes may be up to 8 mm long, so isolation for considerable distances was required. With careful dissection lengths of 4 cm or more could be obtained.

After isolation, single fibres were transferred to a slide on which drops of creosote had been placed. After several fibres had been collected in this manner the creosote was drained off, the slide blotted and the fibres mounted in Canada balsam. Displacement was avoided by using balsam of a low viscosity. The slides were dried in an incubator at 37° C for 1-2 weeks before measurements were carried out.

Measurements were made using an ocular micrometer (Zeiss Compens-Okular, $\times 12$). Those of internodal length were carried out using a $\frac{2}{3}$ in. objective, the draw-tube being adjusted to give a magnification of $\times 100$. Readings of the diameter were taken under a $\frac{1}{12}$ in. oil-immersion objective at a magnification of $\times 1000$. Ten readings were made along each internode, at approximately equal distances apart. By this method it was possible to measure internodal distance to the nearest 10μ and diameter to the nearest 1μ .

ERRORS DUE TO FIXATION AND DEHYDRATION

As the stretches of nerve were attached to cards by both ends throughout fixation and were only removed immediately before teasing, no change in length took place during fixation. With regard to diameter Rexed (1944) found a very slight swelling, not greater than 1.25 %, of the small fibres, after treatment with 4 % formaldehyde, he also reported no change after prolonged storage (up to 4 months) in formalin. Rexed and others have found no diameter change after treatment with osmium tetroxide, although some have reported varying amounts of shrinkage.

To assess the error introduced by dehydration, we measured fibres before dehydration, when in the glycerin and water mixture, again after having been in pure glycerin for 2 hr. and finally after mounting in Canada balsam. Shrinkage occurred mainly in the latter stage and the total amounted to 2.3 ± 0.73 %, affecting length and diameter to the same degree.

TREATMENT OF DATA

For each nerve, the lengths of individual internodes were plotted graphically against the mean of the ten corresponding diameter readings. Further treatment is described in the relevant sections. Where regression lines have been fitted, the method of least squares has been employed.

RESULTS

(1) *The forms of the nodes*

Ranvier (1872) drew attention to the fact that in the ray the myelin tapers towards the node. This is true not only in *Raja* (Pl 1, figs 1-3), but also in *Scyliorhinus* (Pl 1, fig 4) and *Torpedo* (Pl 1, fig 5). The nodes in the conger eel, however, closely resemble those of mammals in the smoothly rounded contours of the myelin, the ends of the two segments appearing as if closely pressed together (Pl 1, fig 6).

Ranvier also reported that the nodal axon in the rays is stained black with osmic acid. In our own preparations there appears to be in most instances a definite interruption of the myelin (Pl 1, figs 1-4), the demarcation of the limits of the sheath being at times very striking (Pl 1, fig 4). At times the region of the node is osmiophilic to some degree, this being most frequently noticed in *Torpedo* (Pl 1, fig 5). It is probable that the axon surface contains a proportion of lipoids similar to

that in non-medullated nerves (Schmitt & Bear, 1939) even where not covered by a thick layer of 'myelin'

The nodes were usually easily recognized and distinguished from accidental breaks in the fibres or from other artefacts

(2) Length of internodes

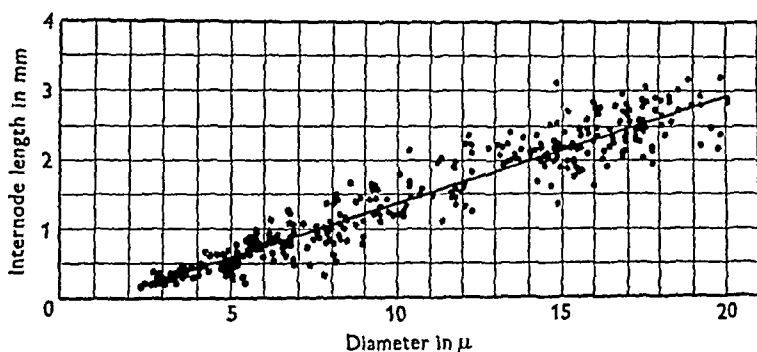
Internodal lengths were found to be even greater than Ranvier supposed. In the lateral line nerves of a ray 83 cm in length and a conger eel 124 cm long the greatest mean internodal lengths found were 7.02 and 7.52 mm on fibres of 22.0 and 26.5 μ diameter respectively, in a dogfish 65 cm long the corresponding value was 4.90 mm on fibres of 19.4 μ . These may be contrasted with the maximum of about 1.5 mm found in the rabbit on the largest fibres (20 μ diameter). Kubo & Yuge (1938) reported lengths up to 3.7 mm in the toad.

The smallest fibres in our series are about 2.5 μ in diameter and have internodal lengths of about 0.20 mm, which is similar to that found in the small fibres of amphibians and mammals (see p. 346).

(3) Correlations between internodal length and diameter

Previous work on this question in fishes has been carried out by Ranvier (1872) and Key & Retzius (1876). The former stated that there exists in the rays a direct relationship, such that the larger fibres have a longer and the smaller a shorter internodal distance. A limited number of observations are given by Key & Retzius for the pike, but their data show little variation of length with diameter.

We have found that there is a positive correlation between internodal length and diameter, and, as in other vertebrates, the relationship is approximately linear.



Text fig. 1. Internodes from lateral line nerve of a small specimen of *Raja* (40 cm in length).

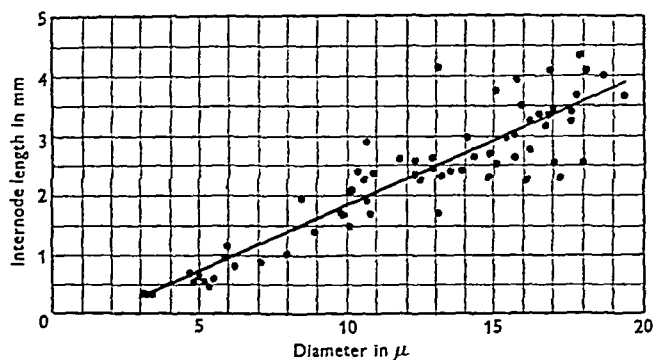
Text-fig. 1 is of fibres from the lateral line branch of the vagus of a specimen of *Raja*, 40 cm long. A regression line has been fitted, and if

$$y = a + bx,$$

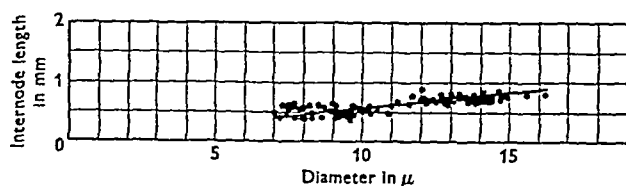
where y represents the internode length in mm, x the average diameter per internode in μ , a the y -axis intercept and b the slope coefficient, a has the value of -0.14 mm and b , 0.15 ± 0.00001 .

Text-fig. 2 gives the measurements from the lateral line nerve of a specimen of *Conger*, 63 cm in length, the constants being $a = 0.38$ and $b = 0.22 \pm 0.0001$.

Text-fig 3 is of the nerve to the electric organ of *Torpedo*. This shows a situation different from that of the lateral line nerves in that there is only a very slight increase of internode length with diameter, $b=0.053$. Moreover, a further peculiar feature is that there are no fibres smaller than 7μ . The significance of this state of affairs is not yet clear, evidently special factors are at work in the morphogenesis of this nerve



Text-fig 2 Internodes from lateral line nerve of a small specimen of *Conger* (63 cm in length)



Text-fig 3 Internodes from electric organ nerve of *Torpedo*

In the nerves of the largest fishes investigated, in the region of the smaller fibres the internodes do not increase in length with the diameter. This is shown in Text-figs 4, 5 and 6, which represent internodes from the lateral line and branchial nerves of the 83 cm ray, and the lateral line nerve from the 124 cm conger eel. Above 5μ the relationship is approximately linear and Text-figs 4, 5 and 6 show regression lines fitted to all fibres larger than 5μ . The significance of this divergence is discussed on p 345, it shows that internode length does not always increase with diameter.

(4) The relationship between internodal length and the length of the fish

This comparison was made using the lateral line nerves of fishes of different sizes. The total length of the fish was used in preference to the actual length of the nerve. To ascertain whether there exists a correlation between body length and internode length, the ratio of the value of the internodal length at the maximum fibre diameter to the length of each fish was employed. The results in the case of two conger eels are set out in Table 1. The standard error is given as a measure of the variance of the mean value of the internodal length at the maximum diameter. It will be seen that each ratio falls within the standard error of the other. Thus it may be stated that for these two nerves

$$\frac{ym}{L} = k$$

The lateral line nerves from three rays yield the data shown in Table 2

Here the ratio is not quite constant, the internodes of the fibres of the largest fish being slightly but significantly longer than expected

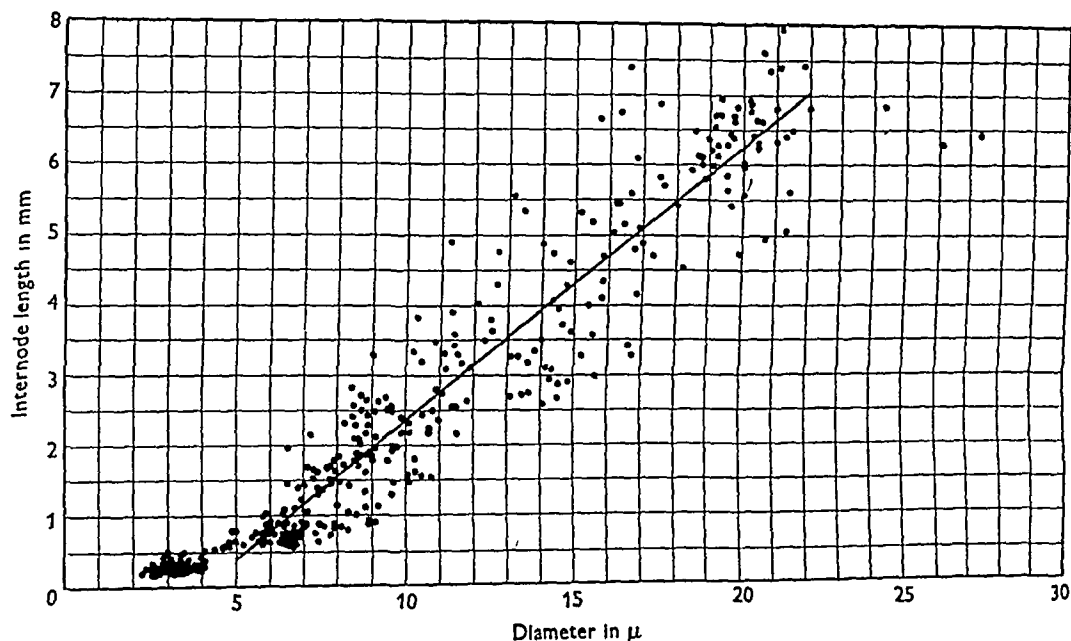
Somewhat similar measurements are given for the ray by Ranvier (1872), these are set out in Table 3. He recorded 'maximum internodal length' and the length of the fish. The ratio of the former to the latter has been calculated in each case and is

Table 1 Relationship between internode length and length of fish in the conger eel

	Length of fish, L (cm)	Internodal length at max. diam., ym (mm)	Int. length at max. diam. length of fish
1	124	7.52 ± 0.20	0.0606 ± 0.00165
2	63	3.84 ± 0.11	0.0609 ± 0.00174

Table 2 Relationship between internode length and length of fish in the ray

	L (cm)	ym (mm)	$\frac{ym}{L}$
1	83	7.02 ± 0.09	0.0845 ± 0.0011
2	40	2.89 ± 0.04	0.0722 ± 0.0009
3	34	2.47	0.0726



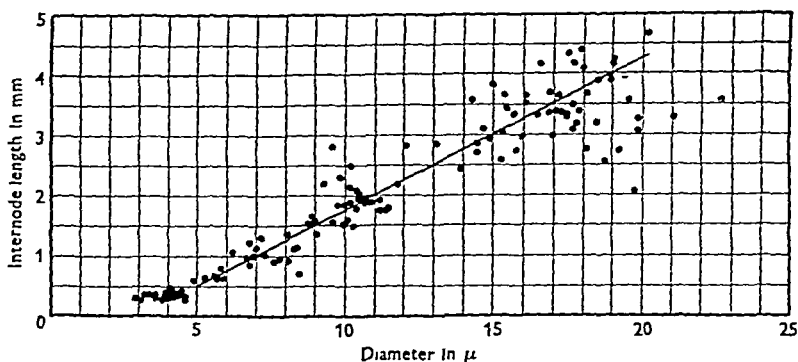
Text fig. 4 Internodes from lateral line nerve of a large specimen of *Raja* (83 cm in length)

approximately constant. The ratios are somewhat lower than those which we have found in *Raja clavata*, this may possibly be a species difference, but unfortunately Ranvier does not state which particular ray he investigated. Alternatively, a different region of the nerve might have been examined.

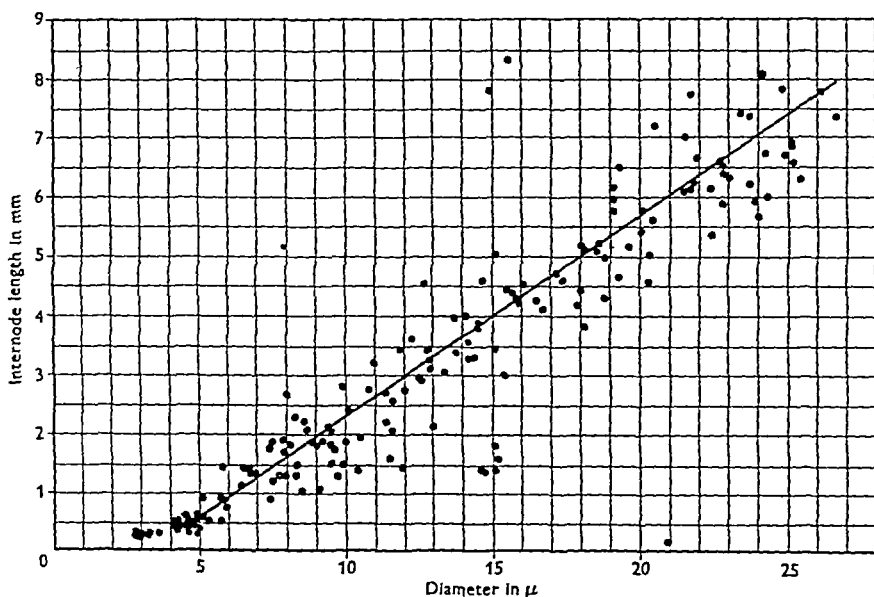
Comparison of maximum internode length with total length certainly supports the hypothesis put forward by study of the nerves of growing mammals that the internodal length is determined by the amount of length increase after the myelin has been laid down. The great length which the internodes may attain in fishes is presumably due to the relatively great amount of growth occurring subsequent to medullation.

(5) Internode lengths in different nerves of the same animal

If growth influences the distance between nodes, then differences should appear between nerves in regions of any one animal growing more, or less, in length, after medullation. Text-figs 6 and 7 show the internode lengths in the lateral line nerve



Text-fig 5 Internodes from branchial nerve of a large specimen of *Raja* (83 cm in length)



Text fig 6 Internodes from lateral line nerve of a large specimen of *Conger* (124 cm in length)

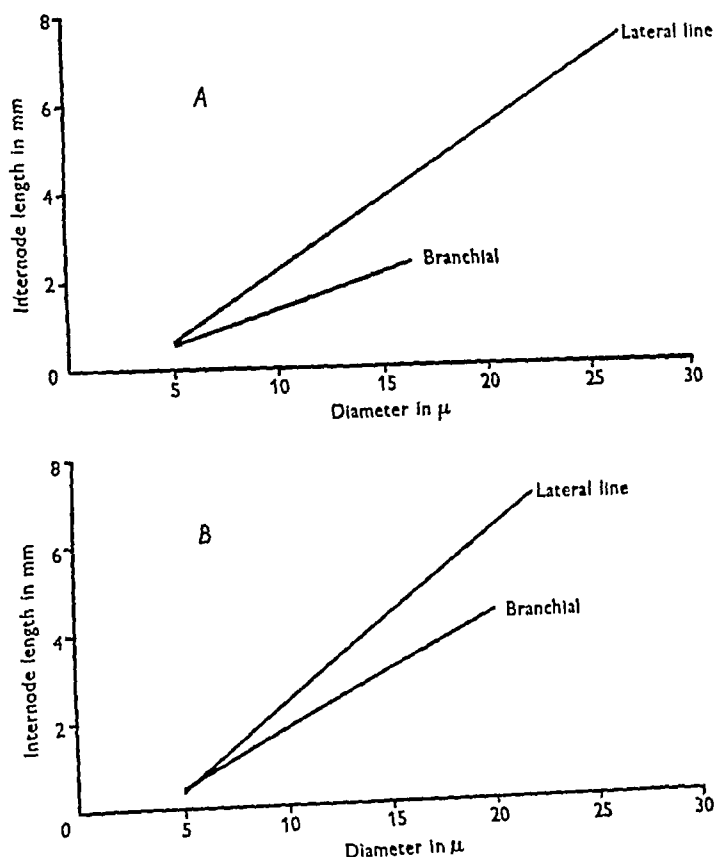
Table 3 Relationship between internode length and length of fish in the ray (data from Ranvier, 1872)

	Length of fish, L (cm)	Max int length (mm)	$\frac{\text{Max int length}}{L}$
1	110	6	0.0545
2	101	5	0.0495
3	36	2.5	0.0694
4	24	1.5	0.0625

therefore, internode length is not solely a function of diameter, a result also strikingly shown in mammals by the fact that after regeneration of a nerve the internodes are all short (Leegaard, 1880, Young, 1945*b*, Hiscoe, 1947, Vizoso & Young, 1948), although the normal diameter spectrum is re-established (Gutmann & Sanders, 1943)

Presumably the proportionality found in normal adult animals between length of internode and fibre diameter is the result of correlation of both with some common factor. We must therefore search for correlation of each with other variables.

The fact that after nerve regeneration in an adult the internodes are short on fibres of all diameters suggests that growth is the factor which controls internode length.



Text fig 9 Regression lines of lateral line and branchial nerves from A, a conger eel 124 cm in length
B, a ray 83 cm in length

(8) Correlation of internodal length with growth of nerves

Ranvier was quick to draw attention to the fact that internodal length is greater in larger than in smaller specimens of an animal. He arrived at this conclusion from measurements carried out on the rays (1872) and the dog and rabbit (1875). This relationship was corroborated by Boycott (1904), Takahashi (1908) and Hatai (1910). The two latter workers noted that internodal length in the nerves to the hind-limb of the frog increases distally, and thought that this was associated with the greater growth rate of the shank as compared with the thigh.

Similar observations have been made by Hiscoe (1947) in the rat, Vizoso & Young (1948) in the rabbit, and now by ourselves in fishes. In the lateral line nerves

examined, internode length was found to be approximately proportional to the amount of growth that the animal had undergone (p 340)

It has been noticed by several investigators that the gradient of the line relating internode length to diameter increases with gain in size of the animal, that is, that the increase of internode length with growth is relatively greater than that of diameter Vizoso & Young (1948), from data obtained from the peroneal nerves of growing rabbits, stated that the slope of the regression lines increased in the same proportion as did femoral length In the present study, we have found the gradient to be greater in the larger animals (Text-fig 8)

The differences between the internode lengths in the lateral line and branchial branches of the vagus nerve may be, as already suggested (p 342), a function of the relative amounts of growth in length that they undergo Again, a similar phenomenon might provide an explanation for the constancy of internode length in the smaller fibres of the largest fishes investigated These fibres are presumably those that have medullated most recently (see later, p 346) If this process occurs throughout the life of the animal, those fibres that become myelinated during the period of the decline in growth rate may increase in diameter in the same way as their predecessors, but fail to undergo the same increase in length

Thus, although the data about growth rates are incomplete, the facts suggest that internodal length is strongly correlated with the amount of growth of the part in which the nerve lies

(9) *The determination of fibre diameter*

If internode length is correlated with growth, can this explain its correlation with fibre diameter? Unfortunately we know very little of the factors that determine diameter, but they are likely to be complicated, since each functional fibre type has its characteristic diameter Diameter is certainly not highly correlated with length Schwalbe (1882) supposed the longest fibres to be the largest, but Dunn (1900, 1902 and 1909) showed that in the frog, the longer fibres are, if any thing, smaller Several lines of evidence, however, show that not all fibres medullate at the same time, and if the ultimately larger ones medullate before the smaller there might be an approximate correlation of diameter with growth

Numerous investigators have found that the appearance of myelinated fibres is prolonged over a considerable part of the growth period in mammals (Hatai, 1902, 1903, Boughton, 1906, Duncan 1934*a*, Corbin & Gardner, 1937, Kjellgren, 1944, Rexed, 1944) On the other hand, Schiller (1889) observed only a very slight increase in the numbers of myelinated fibres in the oculomotor nerve of the cat between newborn and adult animals, but there is reason to suspect that the disparity is the result of the less satisfactory technical methods available to him Birge (1882) and Hardesty (1899, 1900) have obtained similar results in the frog

There is also some evidence of a correlation between time of medullation and ultimate diameter reached by a fibre Boughton (1906), Schumert (1935), Otuka (1910) and Rexed (1944) have all found that the fibres that appear later do not attain the diameter of their predecessors Dissenting are Kiss & Mihálik (1930) who contend that the sympathetic preganglionic fibres in the ventral roots of man medullate before the eventually much larger motor fibres to voluntary muscle

Thus it is probable that the eventually larger fibres become medullated before the smaller, although the detailed relationship has not been worked out. An approximate relationship would be sufficient to account for the correlation of fibre diameter and internode length, which is, after all, not a very close one.

(10) *Absence of increase of number of nodes after medullation*

The hypothesis here adopted implies that the number of nodes is constant for all ages except the most advanced, and this has already been suggested by several investigators. Nevertheless, Vignal (1883*b*) believed that intercalation of new internodes into nerve fibres takes place during growth, and Speidel (1932) has observed *in vivo* the end-to-end anastomosis of adjacent internodes with the obliteration of the intervening node. But Boycott (1904) came to the conclusion that in the frog the 'average internodal distance' increases in proportion to the increase in the length of the whole nerve during growth, and hence that there is no change in the number of nodes. The curve of the average internodal distance plotted against body length followed closely that of the length of the sciatic nerve against body length. This was later confirmed by Takahashi (1908). Hiscoe (1947) and Vizoso & Young (1948) reached essentially the same conclusions in the rat and rabbit respectively. We may assume for the present that in fishes, as in amphibians and mammals, the number of internodes does not increase once they have been laid down, but it would be desirable to have direct evidence of this, especially since the internodal segments are so long in fishes and each may contain several Schwann nuclei.

(11) *When medullation takes place internodal lengths are the same in all fibres*

One of the most striking results emerging from various studies is that there is a minimum internode length that is approximately the same from fishes to mammals. Vignal (1883*a*) reported this to be 0.2 mm. in a foetal sheep. Young (1945*b*) reported it as 0.15 mm. in embryo rabbits, but this may be a little low. Vizoso & Young (1948) found no fibres with internodes below 0.25 mm. in the 2-week-old rabbit. Hiscoe (1947) gives 0.33 mm. for fibres 2.6–4.5 μ in rats 2–4 weeks old, but the nodes may well be a little shorter at the time of medullation in the foetus. Similarly, Ranvier (1875) recorded that the internode length on the largest fibres of the sciatic nerve of the newborn dog is about 0.3 mm.

In the present investigation there is a very sharply demarcated minimum to the internode length data. Thus in Text-fig. 4 it is remarkable that no internode is less than about 0.20 mm. long, the strictness of the determination strongly suggesting the influence of a uniform physical condition. Essentially similar conditions are seen in the younger ray, in *Torpedo*, and in *Conger*.

These facts lend support to the view that this initial periodicity is determined by a limiting physical condition, such as the stable length of a droplet under surface tension (Young, 1944, 1945*a, b*).

Speidel (1932, 1933 and 1935) indicated that the Schwann cells become spaced at approximately regular intervals and that the myelin first appears in the region of their nuclei. Hiscoe (1947) suggested that it is this spacing that is the determining factor and cited in support the observation of Weiss & Wang (1945) that 'the basic length of the Schwann cell in tissue culture is between 200 and 500 μ ', this length

coinciding roughly with the internodal distances of the rats 2-4 weeks old that she investigated. The early development of fish nerves requires elucidation, for whereas in the other vertebrates studied there is but one Schwann nucleus per internode, usually situated near the middle, in the fish nerves we have examined there may be several. Attention has been drawn to this by Key & Retzius (1876). A comparison of the number of nuclei per internode with internodal distance might yield interesting results, the data of Key & Retzius concerning this are too fragmentary to allow of any definite conclusion. In the regenerated nerves of mammals there are also several nuclei per internode.

CONCLUSIONS

Abundant facts are available about lengths and diameters of nerve fibres few tissues have been so satisfactorily studied by quantitative methods. The figures obtained are all consistent with the view that myelin is laid down on young nerve fibres when they reach about $1-2\mu$ in diameter (Duncan, 1934*b*, Schmitt & Bear, 1937). Thus liquid layer does not remain continuous over the fibre, but breaks up into a series of myelin segments, each about 0.2 mm long. A neurilemmal tube is rapidly formed around the myelin, so as to make a chamber containing the liquid.

Normally these myelin segments remain throughout life, each fibre retaining the number of nodes originally laid down. Occasionally internodes may fuse, by disappearance of the intervening neurilemmal inflexions, and also when the axon degenerates and regenerates the original periodicity is lost.

During growth each segment becomes stretched by the tension exerted by neighbouring parts. The neurilemmal sac is closed and more myelin is secreted between the Schwann cell and the axon surface. The length reached by any internode therefore depends on the amount of growth in length of the nerve after it has been laid down.

Since fibres that will ultimately be large are in general laid down early, a relationship appears in a normal adult between diameter and internode length, the latter rising more steeply with diameter in the nerves of those parts of the body in which a relatively greater amount of growth occurs subsequent to medullation.

If the final internode length reached is a function of growth, it is difficult to believe that it is an important variable in controlling conduction velocity or any other functional feature as supposed by Hursh (1939) and von Muralt (1946). The nodes may be fundamentally important as points of high permeability, but their exact spacing seems to have little importance for function. The experiments of Berry, Grundfest & Hinsey (1944) and Sanders & Whitteridge (1946) confirm this by showing that in regenerated nerves conduction velocity increases with the diameter of fibres, although internode lengths remain short.

SUMMARY

1. In the lateral line nerves of the ray and conger eel the distance between the nodes of Ranvier may reach 8 mm. on the largest fibres.

2. Internode length increases greatly with fibre diameter in the long lateral line nerves, but in the shorter branchial nerves there is less increase and in the nerve of the electric organ of *Torpedo* the large fibres have only slightly longer internodes than the smaller.

3 In lateral line nerves from animals of different lengths the ratio of the largest internode length to the length of the animal is approximately constant

4 There is not, therefore, a fixed internode length for each fibre diameter, the correlation between the variables is due to the fact that both are correlated with growth

5 There is a sharply defined minimum internodal length of about 0.2 mm in the various fishes studied, and this is also found in other animal groups

6 Several Schwann nuclei may be found in each internode in fishes

7 The conditions in fishes and other vertebrates are consistent with the view that internode length is determined by surface tension when the nodes are first formed and later by the amount of growth of the nerve. When medullation takes place internode length is short, and is the same for all fibres. As growth proceeds, a relationship between internode length and diameter appears, such that the larger fibres have longer and the smaller shorter internodes. There is little change in the number of nodes after medullation, as the nerves elongate, so too do the internodes. The occurrence of longer internodes on the larger fibres would be explained if the ultimately larger fibres became myelinated earliest.

The complete data, which are too bulky for publication, have been deposited at the Thane Library, University College, London. We wish to express our thanks to Dr F. S. Russell, F.R.S., Director of the Marine Biological Association, Plymouth, where part of this work was carried out, and also to Messrs D. I. Fryer, D. Sholl and A. D. Vizoso for advice and assistance.

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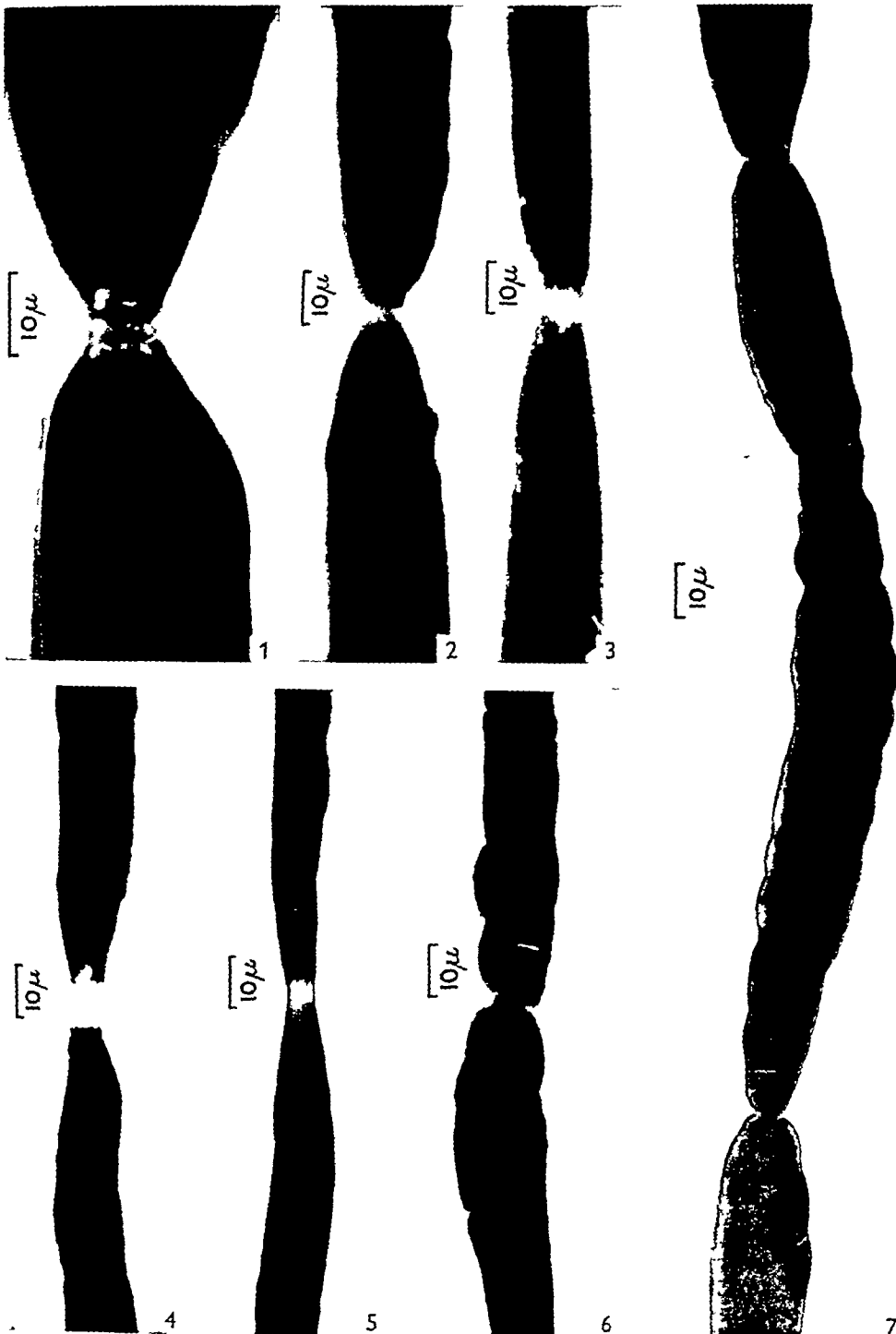
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EXPLANATION OF PLATE

Photomicrographs of isolated nerve fibres stained with osmic acid

Figs 1-6 nodes of Ranvier in *Rana* (figs 1-3), *Scyliorhinus* (fig 4), *Torpedo* (fig 5) and *Conger* (fig 6)

Fig 7 intercalated segment of Renaut in *Conger*



THOMAS AND YOUNG—INTERNODE LENGTHS IN THE NERVES OF FISHES

DIFFUSION PHENOMENON COMPLICATING THE HISTOCHEMICAL REACTION FOR ALKALINE PHOSPHATASE

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INTRODUCTION

The histochemical method for demonstrating alkaline phosphatase in tissue sections, as devised by Gomori (1939) and Takamatsu (1939), has now been widely used for almost ten years, but some of the technical steps are not yet generally agreed upon and the evaluation of the results, particularly as regards precise cytological localization of the enzyme, is, in our opinion, still open to dispute.

Whilst most workers use a similar substrate mixture, either with or without added Mg-ions as enzyme activator, the time of incubation has been subject to wide variations, and many workers remark upon the increased intensity of the reaction that occurs on prolonging the incubation period. The time chosen for incubation would seem to be either quite arbitrary, or that which gives the optimum 'staining' result without loss of contrast between cytological details (Fell & Danielli, 1943, Dempsey & Deane, 1946, Wachstein & Zak, 1946). In other cases, the results of both a short and a long incubation period are recorded and the longer period (e.g. 24 hr. Fell & Danielli, 1943) given preference. The commonest period used is probably 2-3 hr. as originally advocated by Gomori (1939). The depth of 'staining' of the various structures is sometimes graded as black, dark-brown and light-brown and taken to indicate marked, moderate, and slight enzyme activity. If merely the intensity of the reaction were affected by a prolongation of the incubation period, no serious objection could be raised against this variation in the technique. It is, however, frequently stated that structures which are negative or almost so after a short incubation period, become definitely positive if the time of incubation is extended, and this seems particularly to be the case when they happen to lie in sections which somewhere have foci of high and fast reacting phosphatase content (e.g. Horowitz, 1942, Dempsey & Wislocki, 1947). This raises the important question of the localization of the enzyme. Does such delayed reaction signify a weak enzymic activity in these regions or is it due to some 'staining' effect or pseudo-reaction which is brought about by diffusion of some component or other from the true sites of enzyme activity? The existence of such diffusion is also suggested in the literature on the subject by numerous microphotographs—too numerous to be cited individually—which show an obvious gradient of positive cytoplasmic and/or nuclear reactions, often over a wide area, from foci of strong phosphatase activity. If, then, diffusion is at all involved in this histochemical reaction, it might also account for the well-known 'staining' of cells and nuclei (after the standard 2-3 hr. incubation period) at places close to sites of intense phosphatase reaction. Such instances are the internal structures of the epithelial cells of the small intestine or the nuclei of cortical renal tubules and glomeruli. A less familiar example can be observed in the rat pancreas where, not infrequently, the periductular connective tissue gives a strongly positive reaction.

(Jacoby, 1946) and the adjacent duct epithelium shows 'stained' nuclei and a brownish granulated cytoplasm. Is one to speak here of moderate or weak phosphatase activity or of an artifact? The problem seemed to us of sufficiently fundamental importance to warrant investigation by specially designed experiments which might throw further light upon it.

Whilst this work was in progress the papers by Danielli (1946) and by Lison (1948) came to our notice, and both have an important bearing on this problem. Danielli has, in fact, performed experiments similar to, though not identical with, those to be reported here, and arrived at opposite results. He denied that diffusion plays any adverse part in the reaction and affirmed the reliability of the method with regard to the localization of the enzyme. Lison, on the other hand, in his exhaustive review of the histochemistry of phosphatases, frankly states that often some diffusion (i.e. of enzyme) obviously takes place which, in his opinion, is an artifact due to bad fixation.

In this paper experimental proof will be presented to show that diffusion does occur, and that it does so after and not during fixation. There is no evidence that it is connected with bad fixation in the histological sense.

MATERIAL AND METHODS

A General

Except where stated otherwise, all tissues were prepared as follows. They were removed from the animals immediately after these had been killed by concussion and bleeding from the carotid arteries. Small pieces of the tissues, not exceeding a few mm in thickness, were fixed in 80 or 95 % alcohol (usually the latter), dehydrated in absolute alcohol, cleared in xylol, and impregnated with paraffin at 56° C for 2 hr. The whole procedure was always completed within 24–30 hr. Sections were cut at a standard thickness of 7 μ , mounted on slides with a trace of egg-albumen and dried at 37° C, usually overnight.

The histochemical method employed for demonstrating the presence of alkaline phosphatase was a combination of Gomori's (1939) and Kabat & Furtli's (1941) techniques, which was strictly adhered to except for the incubation period which was the main variable.

In view of the importance of the technique in a methodological study of this kind a brief summary of it seems indicated. The paraffin sections mounted on slides were treated in the following way: xylol 1 min, absolute alcohol 1 min, immersion in 0.5 % celloidin (in ether-alcohol \overline{aa}) 2 min, hardening of the celloidin film in 90 % alcohol 5 min, 70 % alcohol, distilled water a few minutes. They were then incubated at 38° C in the following substrate mixture: 3.2 % sodium- β -glycerophosphate 6 ml, 2 % calcium nitrate 9 ml, 10 % sodium barbitone 6 ml, 0.1 M-magnesium sulphate 6 ml, and distilled water 33 ml. The pH of this substrate bath, as tested by means of a universal indicator, is about 9.4. After incubation and a brief rinse in distilled water the slides were placed in 2 % cobalt nitrate for 5 min, washed in three changes of distilled water (occupying about 2 min), immersed in dilute ammonium sulphide 2 min, washed in running tap water 1 min, then dehydrated in the usual way, the celloidin film being dissolved in absolute alcohol or, more speedily, in an ether-alcohol mixture, cleared in xylol and mounted in Canada balsam. No counterstain

was used. Any deviations from this basic procedure which were necessitated for special tests will be referred to in the appropriate places.

Control sections were incubated in a 0.1% calcium nitrate bath, the substrate being omitted; it may be said at this stage that in all instances these proved to be entirely and consistently negative.

B Special experimental procedures and results

We developed two experimental approaches towards the problem at hand. The first consisted of an arrangement in which sections of known high phosphatase content were superimposed on sections known to be negative in this respect. It was thought that, if diffusion is at work in a section with a high enzyme content bringing about a positive reaction (or 'staining') in cells around the sites of high phosphatase activity, such diffusion should also affect a tissue which is placed underneath and that nuclei or cells—which are normally negative—could be made to appear to give a positive reaction.

Guinea-pig liver proved an ideal material to serve as underlying section. Apart from isolated positive leucocytes and/or litoral cells in and along vascular spaces, the liver of guinea-pigs gives no phosphatase reaction in its parenchyma (Pl 1, fig 1), at commonly employed incubation periods (i.e. up to 24 hr). This holds true for paraffin as well as frozen sections. The fact that after excessively long incubation (70 hr) the nucleoli of the liver cells sometimes show a faint 'staining', as Zorzoli & Stowell (1947) have also observed, does not detract from the value of this tissue for the present purpose. Furthermore, this very weak nucleolar reaction of the liver cell nuclei can be completely abolished by heating the section prior to its being used as underlying material.

The large polyhedral liver cells with their characteristic spherical nuclei constitute an easily recognizable tissue even when seen through a superimposed section.

Renal medulla (from rabbits) was also used for the same purpose, its parenchyma is well known to be histochemically phosphatase negative, and we have found that neither paraffin nor frozen sections prepared from it showed any nuclear reaction even after 120 hr of incubation. But structurally renal medulla is not quite so suitable as guinea-pig liver for the purpose of these experiments.

Sections superimposed included duodenum, other parts of small intestine and mid colon, and renal cortex of rabbits, and also small intestine and renal cortex of rats and of guinea-pigs. The heavy positive reaction given by the cuticular border of the small intestine and the convoluted tubules of the kidney need no further comment, but it is worth noting that rabbit mid-colon shows often a strong positive reaction in the tunica propria (affecting practically all the structures of this layer).

In detail, the procedure was as follows. First the 'negative' tissue was mounted and dried on a slide in the usual way. Then paraffin sections were cut from the 'positive' material, and small segments of them floated on to the 'negative' tissue. Care was taken to avoid much water accumulating between the two sections. The double-mounted slide was placed in the incubator and dried overnight. The phosphatase test was then performed in the manner described.

It was found with all these tissues that after an incubation period of 24 hr or more

there was clear evidence of the occurrence of diffusion as reflected in the underlying negative sections. The nuclei of the underlying liver cells near sites of intense phosphatase activity in the superimposed section showed a typical 'positive' reaction. They were not jet-black throughout, but showed intensely blackened nuclear membrane, nucleoli and other chromatin particles (Pl 2, fig 6). There was a definite down-gradient from the sites of intense enzyme activity, so that liver cell nuclei some distance away from these sites showed no reaction at all (Pl 2, fig 5). The same result was obtained with rabbit renal medulla used as the basal negative section.

It was then thought possible, by varying the incubation period, to determine the time at which diffusion could first be detected, the criterion being a 'positive' nuclear reaction in the underlying tissue. Sections from each series prepared were incubated for periods of $\frac{1}{2}$, 1, $1\frac{1}{2}$, 2, 3, 6 and 18 hr. It was found that with enzymatically highly active tissue, such as the striated border of the small intestine, there could be detected a few 'positive' nuclei in the underlying section, very close to the border, in some instances at as short a period as $\frac{1}{2}$ hr incubation (Pl 1, fig 2), but not invariably so (Pl 2, fig 7). At periods of 1 and $1\frac{1}{2}$ hr, diffusion was more clearly evident and more consistent, and became quite obvious and regular at the commonly used periods of 2 and 3 hr (Pl 1, fig 3, Pl 2, fig 8). With times longer than this there was usually a further increase in intensity and spread of the 'positive' nuclear reaction in the underlying tissue (Pl 1, fig 4), gradually approaching a maximum which was sometimes reached at 18 hr incubation.

With paraffin sections of renal cortex as superimposed material diffusion was weaker over the first 3 hr than with small intestine (Pl 3, fig 9), whilst with rabbit colon an intermediate result was obtained, after $1\frac{1}{2}$ hr of incubation many liver cell nuclei lying underneath the positive tunica propria of the colon showed a definite 'positive' reaction. The occurrence of diffusion with this colon material seems of particular interest and significance, for here the enzyme is obviously distributed and situated in a manner and at places different from what is found in the case of intestinal and renal epithelium. This observation therefore suggests that the phenomenon may, indeed, have a wider application.

Furthermore, it should be noted that in these experiments a close parallelism was often found between the appearance and degree of the 'positive' nuclear reaction in the underlying tissue and the spread of the reaction in the superimposed section itself. When, for instance, after 6 hr incubation a superimposed section of small intestine showed positive nuclei throughout its wall, the nuclei of the underlying guinea-pig liver cells were found positive over a similar area, i.e. up to and including those that lay underneath the muscular coats.

The existence of diffusion having thus been established, the question arose whether its occurrence was in any way connected with one or other of the various steps of the method employed and whether it could perhaps be influenced by making certain alterations in the technical procedure. Of these, the covering with celloidin and its subsequent hardening were given special attention because, if the diffusing material was the enzyme itself, these steps seemed to be of particular importance. But neither varying the concentration of celloidin from 0.0 to 1% nor the time of immersion in it (up to 30 min), nor drying it in air (up to 15 min) prior to hardening, nor varying the time of hardening in 90% alcohol (5 min to 6 hr) affected significantly the result.

Diffusion occurred in all instances, but the impression was gained that drying of the celloidin film in air beyond 3 min prior to hardening seemed slightly to reduce diffusion

Omitting the Mg-ions from the incubation fluid, though somewhat weakening the intensity of the reaction and retarding diffusion, did not substantially alter the outcome, especially not after long incubation periods

That the process of embedding was not responsible for the diffusion phenomenon was shown by employing frozen sections as superimposed material Alcohol-fixed renal cortex and small intestine (from rabbit and guinea-pig) were used, some of which had been in absolute alcohol for 4 days, in order to ensure thorough fixation The results were even more impressive than with paraffin sections, the reaction as a whole being more intense, and diffusion occurring readily and at shorter periods of incubation (compare, for instance, Pl 3, fig 10 with fig 9) This is not surprising in view of the higher phosphatase content of frozen compared with paraffin embedded material (Danielli, 1946, Cappellin, 1947)

The possible influence of the time for which the tissue is left in paraffin (at 56° C) during the embedding process was also tested Two pieces of small intestine from a rat were used, one being left in the paraffin bath for 2 hr, the other for 18 hr Sections from each were superimposed on guinea-pig liver and treated in parallel The picture obtained of the phosphatase reaction and the amount of diffusion produced appeared to be about the same in both cases, short and long incubation periods being studied

Whilst examining the slides with sections of small intestine superimposed on guinea-pig liver or renal medulla, we had noticed that within the area corresponding to the lumen of the gut there were, here and there, amorphous masses showing a deep blackening, and areas of liver cells (or renal medullary tubules) underneath and around them also showing a positive nuclear reaction, fading towards the periphery The contents of the small intestine are well known to be rich in phosphatase, and this would account for such a finding It seemed, however, desirable to test this point by special experiments Serial dilutions (with water), ranging from 1/5 to 1/500, were made from intestinal contents, fresh and alcohol-fixed, and a drop of each dilution smeared and allowed to dry on a section of guinea-pig liver or renal medulla The phosphatase reaction was then performed This brought out areas of liver cells (or renal medullary tubules) with a strong nuclear reaction which became weaker towards the periphery of such areas The size and number of these areas decreased with the decreasing concentration of the film of intestinal contents This experiment clearly shows that a positive nuclear reaction can be artificially produced by overlying phosphatase, and the gradient of the nuclear reaction strongly suggests a diffusion phenomenon

Danielli, in his work already referred to (1946), has used as underlying sections rat kidney and spleen, and healing wounds (all containing phosphatase) which had been heated to destroy the enzyme, and frozen sections of alcohol-fixed kidney as superimposed material He was unable to detect any diffusion in significant amounts As his technique, especially the composition of the incubation fluid, differed slightly from ours, we applied his technical procedure to our material and repeated a whole series of the experiments, in some cases also heating the basic section for 40 min at

110° C Again diffusion was apparent in all instances, though the phosphatase reaction as a whole tended to be less intense, due, in all probability, to the absence of Mg-ions which are not included in the substrate mixture used by Danelli

The second experimental approach was designed to find out whether certain parts of a section, which gave a positive reaction only after prolonged incubation, but which were suspected to do so as the result of diffusion from neighbouring areas of high phosphatase activity, would show a positive reaction, if first isolated by microdissection and then subjected to the histochemical test apart from the parent tissue. It was thought possible that such excised material would remain permanently negative and thus, in a negative way, provide further proof for the existence of diffusion.

For this purpose rabbit duodenum was used. With this tissue it had been found that often, on prolonging the incubation period, some or all the nuclei of Brunner's glands and even those of the outer muscle coats became positive, the degree and extent of the reaction varying somewhat from animal to animal. Sometimes only those acini of Brunner's glands that lay towards the lumen of the gut would show a positive reaction, a fact which in itself seemed to suggest a diffusion process.

For these experiments duodena were selected which showed such marked phosphatase activity that after 24 hr incubation or longer nearly all the nuclei of Brunner's glands and the outer muscular coats as well as the usual sites showed a positive reaction (Pl 3, fig 12), whilst after short incubation periods the said nuclei were negative (Pl 3, fig 11). From paraffin sections of such material, floating in a small drop of water on a slide, peripheral wedges were cut out, under a dissecting microscope by means of a von Graefe knife, which were so chosen that they contained only a very few acini of Brunner's glands together with the adjacent muscle coats. Incidentally, these glands are easily recognizable in such floating waxed sections, appearing greyish black in transmitted light. The need for very small wedges will be obvious from reference to Fig 11 of Pl 3, which shows that, after only $\frac{1}{2}$ hr incubation, there can be found two sites of strong phosphatase activity—the inner border of the lining epithelium and patches of tissue amongst Brunner's glands. It is difficult to decide on the nature of this tissue, but it appears, in part at least, to be composed of capillaries. Inclusion of any such patches in the excised wedges might, naturally, obscure the results. However, by excising and testing such wedges in sufficient numbers, this danger can be avoided. The isolated wedges and the parent sections, from which they had been removed, were then mounted on separate slides, dried and treated in the usual way, wedge and 'donor'-section being incubated simultaneously in the same substrate solution.

Such suitably isolated wedges remained negative when incubated for periods ranging from 24 to 72 hr (Pl 4, fig 13), whilst the parent sections and also whole neighbouring serial sections gave a generalized nuclear reaction in all tissues, including Brunner's glands and the outer muscle coats. Fig 14 of Pl 4 shows a reconstructed picture of a parent section with the wedge that had been excised from it and incubated separately.

ADDITIONAL OBSERVATIONS

Having found that diffusion may become evident after only $\frac{1}{2}$ hr of incubation, we thought it would be interesting to study the results of even shorter incubation periods. Without going into details of the various tissues thus investigated, it may suffice to put on record here that in the case of rabbit small intestine an incubation period of 2 mm only was enough to bring out a strong reaction of the striated border of the epithelial cells, the remainder of the section being entirely negative (Pl 4, fig 15).

A further observation was made on the duodenum (also of rabbit). Its covering epithelium commonly shows, in places, a positive reaction in the supranuclear region which, as Emmel (1945) has shown (especially in the mouse), is identical in position with the Golgi zone, and various suggestions have been made with regard to the functional significance of the presence of the enzyme here in relation to the supposed activity of the Golgi apparatus. We found that incubation for 20 min produced a strongly positive reaction in the intestinal border of the epithelium and a weaker, but definite reaction in the Golgi region of many of its cells, whilst the nuclei and the remaining cytoplasm were practically negative at this period (Pl 4, fig 16). This finding might indicate that the Golgi zone has an inherent, independent phosphatase activity. Alternatively, it might suggest that its affinity for whatever diffuses from the heavily reacting border is slightly greater than that of the nuclei. In view of the ease with which nuclei can be made to give a positive reaction in the close vicinity of foci of high phosphatase activity, we are, at present, inclined to accept the positive reaction of this Golgi region as a genuine one.

DISCUSSION

The experiments described demonstrate that a process of diffusion interferes in the standard histochemical methods (Gomori-Takamatsu technique) used for showing the presence of the enzyme alkaline phosphatase, in such a way that it will produce an apparent positive reaction in structures, especially nuclei, that contain no inherent histochemically demonstrable alkaline phosphatase. Such diffusion is particularly favoured by the presence, somewhere in a histological section, of foci of high phosphatase activity. The longer the incubation period, the more likely is diffusion to occur, and our experiments indicate that, at least for certain tissues, the incubation periods commonly employed, i.e. 2-3 hr, are too long if precise localization of the enzyme is to be determined, whilst periods of 20-24 hr, which have been advocated must, at least for many tissues, be regarded as excessive from this point of view. If no more than an appraisal of enzyme activity is to be made, without the aim towards its exact localization, then there is no disadvantage in using long periods of incubation, in fact these might be favourable for making comparative estimates of enzyme activity in different tissues.

It will also have become apparent that an estimation of all sites of enzyme activity in a histological section is a difficult matter, and is perhaps in many cases impossible with the standard technique. Diffusion will first occur from the sites of most intense phosphatase activity with the result that not only inherently negative structures close to these sites might show an apparent positive reaction, but it may also affect and mask sites of weaker, but true enzyme activity. The latter sites may indeed need a longer incubation period to be revealed. They can in this way be evaluated,

but only if they lie well away from regions of intense enzyme activity and the intervening tissues show no reaction. The limitations of the method are thus obvious, and discretion must be exercised in an assessment of the localization of the enzyme. It is, for instance, open to doubt whether the nuclei of the lining epithelium of the small intestine have histochemically an inherent phosphatase activity, a point to which we shall return later.

To overcome or, at least, to reduce these difficulties we feel justified, if and when exact localization of the enzyme is aimed at, in recommending first of all omission of the Mg-ions from the incubation mixture, as is actually done by a number of workers, secondly, drying the celloidin film in air for 3-5 min prior to hardening in 90% alcohol, though this point needs further testing and confirmation, thirdly, to perform, as a preliminary experiment, such procedure as superimposing small sections of the tissue under investigation on paraffin sections of guinea-pig liver in order to determine the maximum incubation period which can safely be used without the result being complicated by diffusion.

In order to find out whether certain parts of a section have a weak enzyme activity that is being masked by diffusion, it might, in favourable instances, be possible to settle the point by an isolation experiment comparable with that which we have carried out on the duodenum, but it will not always be an easy technical procedure, and even micromanipulation may have to be resorted to.

Future tests of these kinds will reveal the range of the validity of our observation, for the number of tissues investigated so far has been naturally limited. But it is worth noting that in these tissues which showed the occurrence of diffusion, the enzyme is in all probability situated in extra- or near-extracellular sites. This holds for the striated border of the small intestine, the brush border of the convoluted renal tubules (see Danielli's remarks on this point, 1946), and probably also for the tunica propria of rabbit mid-colon and similar sites in other parts of the intestine where fine reticular fibres are usually amongst the first structures to react. In the loose connective tissue surrounding Brunner's glands the reaction is often related to blood capillaries, where it might be due to the phosphatase of the blood itself. On the other hand, there seem to exist sites containing the enzyme, under the action of which diffusion does not occur, or at least not readily. This is, for instance, the case with the individual cells in and along the sinusoids of guinea-pig liver which show an intense reaction, but there is no spread to the nuclei of the neighbouring liver cells. In these cells, most of which are probably leucocytes, the reaction is strictly intracellular, affecting predominantly the cytoplasm.

As to the pertinent question of what constitutes the diffusing material, whether the enzyme itself or its reaction product, our experiments do not permit of a definite answer. But some observations and considerations should be made on this question. Danielli, when putting this problem to an experimental test, assumed that calcium phosphate would be the diffusing material, as he had observed that nuclei have a high affinity for calcium phosphate when this is precipitated in a section by non-enzymic procedures. As already stated, Danielli in his experiments with superimposed sections did not observe such diffusion. We are unable to account for this, but it may be that his choice of material, especially renal cortex on heated renal cortex, may have been an unfortunate one and must have presented considerable difficulty in evaluation.

However, it must be added that in some cases Danielli had evidence of diffusion, but this occurred when the superimposed frozen material had come from 80 % alcohol and had not been taken into absolute alcohol. The phenomenon was, therefore, considered to be due to diffusion of the enzyme itself from inadequately fixed tissue. This links up with Lison's contention, to which reference has already been made. Our material does not show signs of bad fixation in the histological sense. As to the fixation of the enzyme in tissue sections, little is known. Lison was the first of the histochemists to draw attention to the fact that the enzymes (i.e. phosphatases) are present in the tissues in a lyo- and a desmo-variety. He points out that it is only the latter which can be and is detected histochemically.

However, he does not make it clear which enzyme variety is involved in the diffusion phenomenon. There seem to exist, at least, two important possibilities. (a) The lyo-enzyme variety has not been completely lost in the process of fixation, embedding, etc., and such as is left behind gradually diffuses, whenever the material passes through watery solutions, into the surroundings where it becomes anchored on suitable surfaces or interfaces, especially such as are presented by cell nuclei, it then becomes active here and causes the precipitation of the phosphate. If this is the correct interpretation of the diffusion phenomenon, inadequate loss rather than bad fixation of enzyme would be responsible for it, and for precise localization an optimum loss of enzyme has to be aimed at—a rather precarious goal!

(b) If, on the other hand, the lyo-enzymes have, as is supposed, been completely eliminated prior to incubation, then the phenomenon needs another explanation. The desmo enzymes, by definition, are held in their places. Here they are active and as a function of time and their own quantity produce increasing amounts of calcium phosphate. It is conceivable that not all the calcium phosphate formed will remain, or even find room, at the site of enzyme action, but that with time some will diffuse away and settle on other structures which have, for some reason or other, a high affinity for it. Nuclei, as Danielli has shown, are such structures. It is then visualized here in the usual way and produces the apparent positive nuclear reaction.

Menten, Junge & Green (1944*a, b*) have described a different technique for the histochemical demonstration of alkaline phosphatase by precipitating and visualizing the organic radical of a suitable substrate (calcium- β -naphthyl phosphate) in the form of an azo-dye. This method has been criticized because of its failure to demonstrate nuclear phosphatase in such epithelial cells as those of the convoluted renal tubules (Lorch, 1947). May it not be that, on the contrary, the enzyme localization with this method is more accurate and less complicated by diffusion? Perhaps the reaction product is here not easily adsorbed on to nuclear structures. In other words, these observations might be used for arguing that in the Gomori-Takamatsu technique the calcium phosphate—and not the enzyme—is the diffusing material.

Apart from these two main possibilities considered so far, a further alternative exists. It may be that many, if not all, nuclei actually contain the enzyme, but in an inactive form, and what diffuses is a substance counteracting this inactive state, either an activator itself or an inhibitor-removing substance. Such hypothesis is particularly attractive in view of the important role the enzyme is supposed to play in the nucleic-acid metabolism of nuclei. It would also solve a discrepancy which exists between the results of chemical analysis of isolated nuclei (see below) and

histochemical evidence with which we are concerned exclusively in the present investigation. Attractive as this idea is, our results with heated guinea-pig liver as underlying material, which still showed the diffusion effect, precludes its acceptance. It will, thus, be obvious that further experimentation is necessary to decide which of the various possible explanations holds true and will account for the diffusion phenomenon.

It is not within the frame of this paper to discuss the problem of nuclear phosphatase. On chemical analysis of isolated nuclei, as just indicated, the enzyme has been frequently, if not invariably, found, but the number of types of nuclei thus investigated is still restricted. On the other hand, it is certain that histochemically the resting nuclei of numerous tissues are consistently phosphatase negative. To what extent, then, can we trust histochemical evidence? The answer is usually given in the following form: a positive result shows the presence of the enzyme, but a negative result does not necessarily demonstrate its absence (Cappellin, 1947, Lison, 1948). In the light of our work this conception needs some amendment. Though a positive reaction doubtless indicates the presence of the enzyme, it does not necessarily give its precise localization, nay, may even mask histochemically inherently negative structures. As to a negative result, this may well demonstrate the absence of the enzyme under the conditions under which the tissue was examined. Such a negative result should be compared, as to its validity, not with the enzyme content of fresh tissue as assessed by chemical means, but with biochemical tests made on material treated in the same way as the section. Only a discrepancy in the latter comparison would invalidate a histochemically negative result.

Finally, the implication of this work with regard to the existing literature on the subject has to be briefly considered. It will be obvious that some work which has been done in the past will need some revising and amending. It is impossible to enumerate all the instances in the literature in which the results and interpretations are clouded by this diffusion phenomenon. In most cases positive nuclear reactions are reported without qualification, regardless of their situation in a section and possible vicinity to foci of high enzyme content. Only very occasionally the word 'apparent' has been added. Yet a few examples, chosen at random, might be instructive. Thus Horowitz (1942), investigating rat foetal heads with loci of high phosphatase activity, which are brought out within a few hours of incubation, states that, if incubated long enough, practically all tissues will show the presence of the enzyme. This, according to the author, agrees with chemical investigations which have shown that in most mammalian tissues the enzyme can be detected (but compare our earlier remarks on this point). Wislocki & Dempsey (1946), in their bone marrow studies, describe a positive reaction of the endothelium of vascular channels and of reticular fibres and also of the neighbouring (*sic*!) developing blood cells. The same authors (Dempsey & Wislocki, 1947), investigating various mammalian placentas, which give a strongly positive reaction at the border of the chorionic epithelium, report that the nuclei of the mesenchymal cells in the cores of the villi, though negative after 3 hr incubation, are often positive in sections incubated beyond 3 hr. Again, Atkinson & Elftman (1947), in their interesting study on the increase of phosphatase activity in the mouse uterus under oestrogenic stimulation, remark that the enzyme content of the nucleus (*i.e.* of the epithelium) appears to

rise with the increment in cytoplasmic alkaline phosphatase, and also that, after prolonged stimulation, the amount of phosphatase in the periglandular stroma appears to be noteworthy

The situation is more complex in such studies as Emmel (1946) carried out on the differential inhibition of alkaline phosphatase by potassium cyanide. He found, amongst other things, that the nuclear phosphatase of the intestinal epithelium (rat) is already inhibited at a lower concentration of KCN than that of the cuticular border (incubation period 3 hr). There is as yet no definite evidence that these nuclei have histochemically an inherent phosphatase activity at all. They will always be positive at the commonly employed incubation period of 2-3 hr, and might well be so as the result of diffusion. If this is so, then Emmel's findings would not indicate a differential inhibition, but merely a graded suppression of the phosphatase at the border which, at the lower concentration of KCN, will itself still give a positive reaction, whilst its activity is sufficiently inhibited so as to prevent diffusion from occurring.

To establish whether or not these nuclei of the villus epithelium harbour histochemically an inherent phosphatase seems a rather important task. This can be illustrated from yet another context pertaining to the relationship of nucleic acids and alkaline phosphatase. Brachet & Shaver (1948) treated sections of alcohol-fixed mouse intestines with desoxyribonuclease. The nuclei then became unstainable with toluidine blue nor would they give a positive Feulgen reaction, but Gomori's alkaline phosphatase reaction remained very intense. The authors take this as evidence for the dissociation of desoxyribonucleic acid and phosphatase in support of Jeener's work (1946) on isolated red cell nuclei. Such evidence and interpretation can only be accepted if and when it will have been shown that diffusion from the striated border is not responsible for the 'positive' reaction given by the nuclei of the intestinal epithelium.

These examples, selected from the literature, may suffice to show the complications which arise as a consequence of this diffusion phenomenon occurring in this otherwise most valuable histochemical test for alkaline phosphatase, and their discussion might be helpful in indicating future lines of research.

SUMMARY

1 An investigation has been made to determine the reliability of the standard (Gomori-Takamatsu) histochemical technique in demonstrating the precise localization of the enzyme alkaline phosphatase.

2 Two different experimental approaches are described, the purpose of each being to determine whether or not diffusion occurs as a complication during the standard histochemical procedure.

3 It is shown that a process of diffusion does in fact occur during incubation of the sections, leading to an apparent positive result in structures that contain no inherent histochemically demonstrable alkaline phosphatase.

4 The degree and extent of the diffusion is roughly proportional to the incubation period and is often quite marked after the commonly employed incubation periods of 2-3 hr.

5 The implications of these findings are discussed.



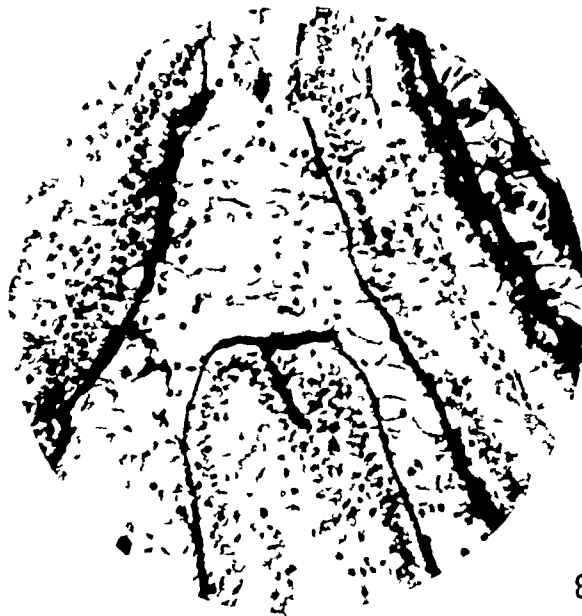
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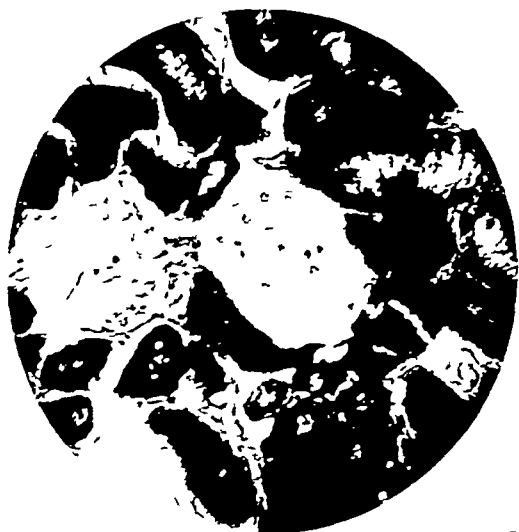
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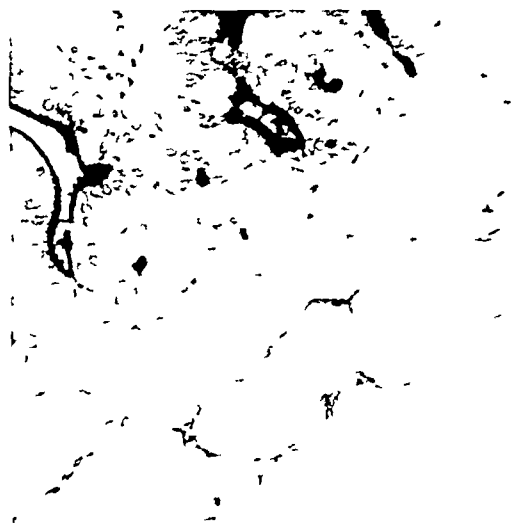
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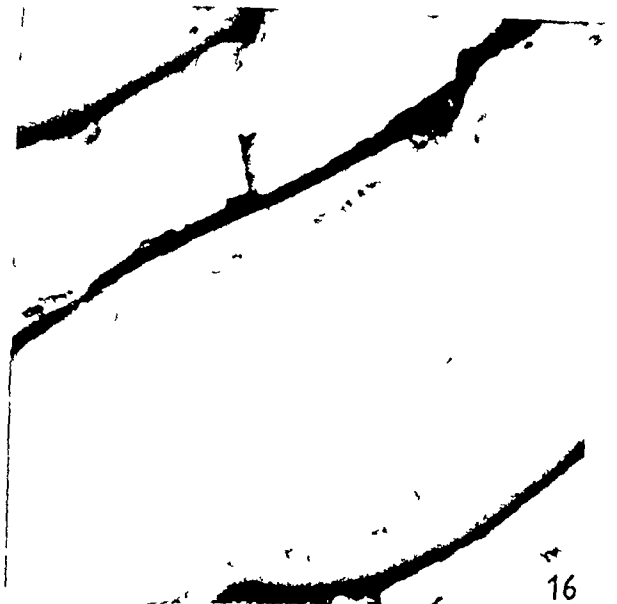
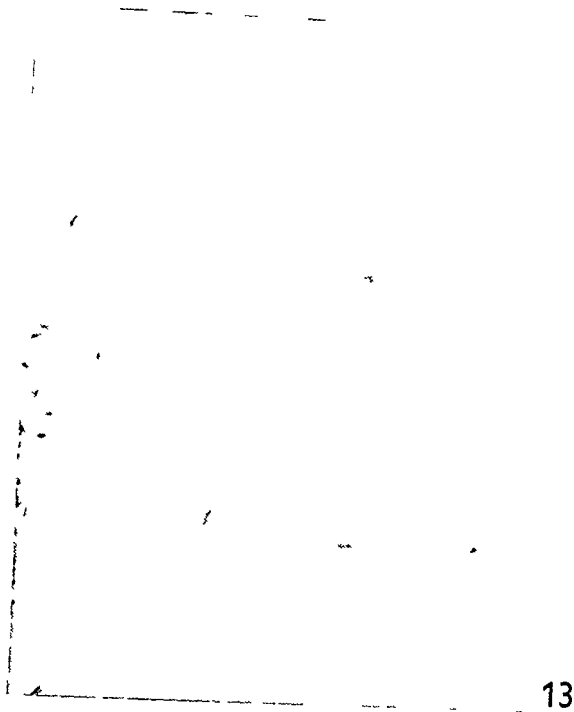


PLATE 1

- Fig 1 Guinea pig liver 24 hr incubation Note that except for a few blackened isolated cells in sinusoids the tissue is entirely negative $\times 190$
- Fig 2 Rabbit duodenum on guinea pig liver $\frac{1}{2}$ hr incubation Strong reaction of the striated border, of some intestinal contents and again some positive cells in the liver sinusoids The nuclei of the intestinal epithelium and also some liver cell nuclei (upper left hand corner) are just starting to give a faint positive reaction $\times 190$
- Fig 3 Rabbit duodenum on guinea pig liver 3 hr incubation Note in this and the following three photographs the increasing reaction given by the nuclei of the intestine and the underlying liver cells $\times 190$
- Fig 4 Rabbit duodenum on guinea pig liver 6 hr incubation Note decreasing gradient of the hepatic nuclear reaction towards the upper left-hand corner $\times 190$

PLATE 2

- Fig 5 Same preparation as that of Fig 4 $\times 130$, to show the gradient more clearly
- Fig 6 Rabbit duodenum on guinea pig liver 24 hr incubation $\times 190$
- Fig 7 Rabbit duodenum on rabbit renal medulla $\frac{1}{2}$ hr incubation All nuclei practically negative $\times 190$
- Fig 8 Rabbit duodenum on rabbit renal medulla 3 hr incubation Note strong nuclear reaction not only in the intestine, but also in many of the underlying renal tubules near to the intestinal border $\times 190$

PLATE 3

- Fig 9 Rabbit renal cortex (paraffin section) on guinea pig liver 3 hr incubation Note faint, but definite positive nuclear reaction in the liver cells in the centre of the photograph $\times 190$
- Fig 10 Rabbit renal cortex (frozen section) on guinea pig liver 3 hr incubation Note strong positive nuclear reaction of the underlying liver cells Compare with Fig 9 $\times 190$
- Fig 11 Rabbit duodenum $\frac{1}{2}$ hr incubation Brunner's glands and outer muscle coat practically negative But note the few positive connective tissue patches between the glands $\times 280$
- Fig 12 Rabbit duodenum (same tissue as shown in Fig 11) 48 hr incubation Note heavy positive nuclear reaction in Brunner's glands and also in the muscle $\times 280$

PLATE 4

- Fig 13 Excised wedge from section of rabbit duodenum, containing Brunner's glands and outer muscle coat (same material as shown in Figs 11 and 12) 72 hr incubation Result reaction entirely negative $\times 280$
- Fig 14 Reconstructed picture with excised wedge and parent section Both were incubated simultaneously, but separately for 24 hr $\times 200$ approx
- Fig 15 Rabbit small intestine 2 min incubation $\times 190$
- Fig 16 Rabbit duodenum 20 min incubation $\times 580$

THE DEVELOPMENT OF MOUSE HAIR *IN VITRO* WITH SOME OBSERVATIONS ON PIGMENTATION

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INTRODUCTION

In the past much attention was paid to the development and structure of hair, and more recently interest has turned to the effects of nutrition and climate on hair growth. Although very many factors have been shown to affect hair growth, little is known of their mode of action, and a number of reports are conflicting. There is need for further study of the interplay of heredity and environment and for an analysis of systemic and local effects on hair differentiation and growth.

Skin grafting has been used by many workers to study the physiology of hair growth. Reed & Sander (1937) employed the special technique of grafting embryo mouse skin on to newborn mice. Butcher (1946) transplanted pieces of the skin of young rats either subcutaneously or into the peritoneal cavity of the donor animals without interrupting the vascular or nervous supply.

There have been several attempts to alter the environment of hair follicles more thoroughly by making grafts to the developing chick. Hiraiwa (1927) showed that pieces of rat embryos underwent differentiation on the chorio-allantoic membrane of the chick, and claimed that normal hair follicles were developed. However, Nicholas & Rudnick (1933), using the same technique, were unable to confirm his results for epidermal structures. Reed & Alley (1939) sometimes obtained differentiation of hair follicles from embryonic and newborn mouse skin on the chick chorio-allantois. Normal hair follicles differentiated in grafts of embryonic mouse tissues in the chick coelom made by Rawles (1940, 1947).

Hair follicles were reported by Waterman (1940) in some of his grafts from mouse embryos to the omentum of the adult rabbit. Grafts of rabbit and rat embryo tissues to the adult rat kidney and to the adult rabbit kidney and omentum were much less successful than grafts to the chorio-allantoic membrane of the chick (Waterman, 1936).

All of the above techniques expose the skin and hair follicles to a blood-vascular system in which the volume and composition of the nutrient fluid is continually changing. They are therefore unsuited to analytical studies of the effect of nutrition and environment on hair development and growth, although they have proved very useful for other purposes as in the study of pigmentation and some hereditary factors.

The object of the present work is therefore to develop a method for studying hair differentiation and growth in the absence of a circulatory system. Such a method could be used to examine the effect of nutrition, temperature, light and other radiations acting directly on the skin, without confusion from systemic effects. To the knowledge of the writer only three attempts have been made previously

to grow hairs *in vitro*, and of these only two have been published as short notes Strangeways (1931) obtained some differentiation of hair follicles when the skin of embryo guinea-pigs was grown in tissue culture, but she was more successful with the vibrissae of the snout than with ordinary pelage hairs from the trunk Murray (1933*a, b*) made some unpublished observations on hair-follicle, differentiation *in vitro* from embryo rat skin, and found that while vibrissae developed normally, the follicles of pelage hairs were unable to do so Rawles (1940) referred in a footnote to a few trial tissue cultures of embryonic mouse skin made by Mr H L Hamilton, and reported that hair follicles differentiated, but gave no details

The present paper describes the complete differentiation of hair follicles and the growth of hair in explants of embryonic mouse skin cultivated *in vitro*

MATERIAL AND METHODS

Most specimens of the house-mouse, *Mus musculus*, were from the laboratory stock of albinos, two were from a mixed stock with grey-bellied agouti, or 'wild-type' colouring, and one was a mouse of the C57 Black strain Embryos from 10 to 18 days post-coitus were used The ages were calculated to the nearest day from the time of mating, indicated by the vaginal plug, and were checked by Gruneberg's (1943) description of the external features In the case of embryos of 13 days or more, the vaginal bleeding served as an additional check One, two or three members of a litter were used in each experiment, and pieces of the skin were fixed for controls

The culture medium consisted of equal parts of fowl plasma and chicken-embryo extract The extract was prepared by adding two volumes of Tyrode solution to one volume of a minced chicken embryo between 10 and 12 days old The explants were transferred to fresh medium every 3-6 days

Nine series of hanging-drop cultures were made from the dorsal and lateral surfaces of the trunk, and occasionally the ventral surface also was used With the aid of a low-power binocular microscope, a sheet of ectoderm with the underlying mesoderm was removed from the body with fine cataract knives This sheet was cut into squares with a side of about 1.5 mm, and each square was placed on the surface of a clot of plasma and extract, with the mesoderm next to the clot The cultures, on $1\frac{1}{4}$ in square cover-slips, were inverted over $3 \times 1\frac{1}{2}$ in hollow-ground slides and incubated at 34 or 37°C Living cultures were examined under the microscope, individual follicles could be clearly seen and their progress recorded from day to day It was often possible to distinguish the individual cells composing a follicle and to follow their differentiation

In the three series of cultures made by the watch-glass technique, larger pieces of tissue were cultivated in a greater volume of the medium than was used for the hanging-drop method In Exps 10 and 12 each explant consisted of the dorsal, lateral or ventral skin of one side of the trunk, and frequently included also portions of the underlying ribs, sternum or vertebrae In the cultures from 10-day embryos of Exp 11 each explant consisted of the entire dorsal or ventral half of the trunk The watch-glass cultures, which were incubated at 37°C, were examined daily, the general state of hair follicle development was noted, but details could not be seen as clearly as in the hanging-drop cultures

Cultures were fixed at intervals throughout each experiment, and usually some were continued until healthy growth ceased. In the twelve experiments 233 explants were cultivated. These and the controls were all fixed, usually in Zenker's fluid with 3 % acetic acid, they were embedded in paraffin and complete serial sections were cut at 8μ . Staining was with Mayer's haematoxylin, chromotrope and picric acid, or with Heidenham's Azan.

Normal hair development was studied from the tenth day of embryonic life up to the adult condition. The normal skin fragments were fixed in Zenker's fluid with 3 or 5 % acetic acid, embedded in paraffin or in ester wax by the method of Steedman (1947), and cut and stained in the same manner as the cultures.

Living cultures and serial sections from both cultures and normal skin were examined with a polarizing microscope to observe the birefringence of hairs and other structures.

NORMAL DEVELOPMENT OF MOUSE HAIR FOLLICLES

Among the many good histological descriptions of the development of hair in mammals are one by Segall (1918) for the guinea-pig and one for the rat by Danneel (1931). Mauier (1892) figured two stages in the development of hair follicles in the mouse, and Oyama (1904) defined and illustrated four stages. In a study of pigmentation Hentschel (1930) figured the early and late stages of follicle differentiation found in the first few days after birth. Gibbs (1941) described the development of mouse hairs and follicles during the first 15 days after birth, and reported that the pre-natal development was similar to that in the Australian opossum, *Trichosurus vulpecula* (Gibbs, 1938). Dry (1926) studied the hairs of mice from birth to 4 months by dissection of the follicles.

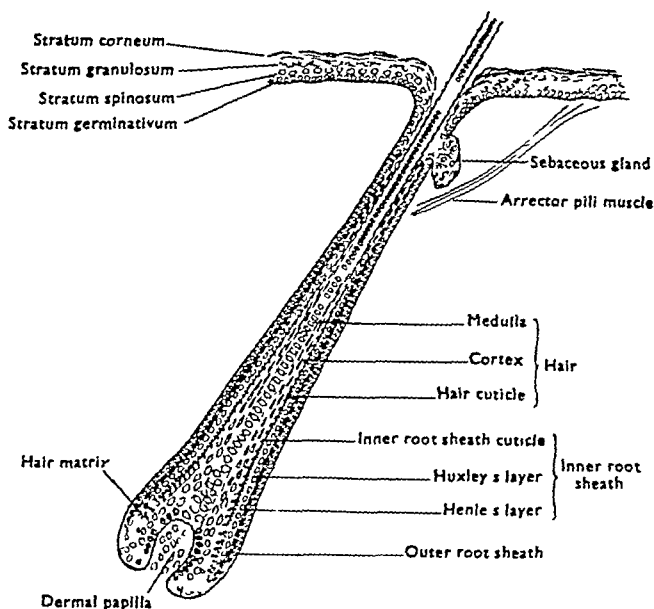
None of these authors correlated pre-natal hair follicle development in the mouse with changes in the skin or with the age of the embryos. A summary is therefore given of the main stages of development found in the present material. The summary is continued until the end of the first 4 weeks after birth and is correlated with the observations of Gibbs (1941) and Dry (1926). There was considerable variation in the length of the gestation period, but for this summary it is arbitrarily chosen as 21 days, and post-natal ages are recorded as the number of days from birth, prefixed by a 'plus' sign. The terminology for the mouse epidermis is that adopted by Hanson (1947). Reference is made to the measurements by Gibbs (1941) of the thickness of the epidermis and of the derma-plus-hypodermis. The names of the hair-follicle types are those proposed for mammals in general by Wildman & Carter (1939), but reference is also made to the terms used by Gibbs and by Dry. Since there are regional differences in the rate of hair development, the age recorded for each stage applies only to the lateral trunk region.

The adult mouse has no sudoriferous glands, the transitory sudoriferous gland rudiments reported by Gibbs (1941) were not seen. The main features of the structure of an active hair follicle are illustrated in Text-fig 1.

Stage 0 No follicles 12-13 days. The epidermis consists of stratum germinativum and periderm, sometimes also with a stratum intermedium one to two cells thick. No hair follicles are present. The derma is not differentiated from underlying meso-

derm Blood vessels and nerves are present in the deeper part of the presumptive derma (Pl 1, fig 1)

Stage 1 Follicle plugs 14 days (Haarkeim stage of Oyama) Plugs of epidermis extend into the mesoderm by the rapid division of cells of the stratum germinativum There is a condensation of dermal cells at the base of each plug These are the primary X follicles (primary follicles of Gibbs, monotrichs of Dry) The epidermis consists of stratum germinativum, stratum intermedium and periderm



Text fig 1 Diagram of a fully developed mouse hair follicle

Stage 2 Pre-papillae 15 days The bases of follicle plugs flatten prior to invagination The epidermis consists of stratum germinativum, stratum spinosum and periderm Cells of the panniculus carnosus muscle make their first appearance (Pl 1, fig 2)

Stage 3 Papillae 16 days (Haarzapfen stage of Oyama) The bases of the follicle plugs are invaginated and rounded dermal papillae are formed The epidermis has stratum germinativum, stratum spinosum, stratum granulosum and periderm (Pl 1, fig 4)

Stage 4 Fibre cones 19 days (Bulbuszapfen stage of Oyama) The dermal papillae are longer than they are wide In each follicle the elongated hardened cells of Henle's layer form a cone pointing toward the skin surface The epidermis forms a stratum corneum (at 18 days)

Stage 5 Hair canals 20 days A space appears in the stratum spinosum and granulosum above each developing hair follicle A slight swelling at one side of the follicle marks the position of the future sebaceous gland (Pl 2, fig 9)

Stage 6 Hair formation 21 days, or at birth (Beginning of Scheidenhaar stage of Oyama) Inside the fibre cone of Henle's layer are distinguished the cells of

Huxley's layer, the inner root sheath cuticle and the tip of a hair fibre with a cuticle and cortex. Pigment is seen in the epidermal cells of the 'hair matrix' in some follicles. The epidermis is thick. Connective tissue fibres form a reticulum in the derma. Between the derma and the panniculus carnosus is a hypodermis of loosely arranged connective tissue with fat cells and blood vessels (Pl 1, fig 7, Pl 2, fig 9).

Stage 7 Hairs in hair canals +1 day The tips of the first hairs have reached the hair canals. Sebaceous cells appear in the sebaceous gland swellings. Primary Y follicles (secondary follicles of Gibbs, awls and auchenes of Dry) appear between the primary X follicles as epidermal plugs. The epidermis and the derma-plus-hypodermis are both increasing in thickness (Gibbs).

Stage 8 Hairs emerged +2 to +3 days The tips of the primary X follicles emerge from the skin. The primary Y follicles are differentiating (Gibbs). The epidermis and derma-plus-hypodermis are increasing in thickness (Gibbs). The periderm and outer layers of stratum corneum are shed while the hairs are emerging (Hanson).

The above stages refer to the complete differentiation of the primary X hair follicles, while stages 9-13 are concerned with the formation of primary Y and later follicles.

Stage 9 'Trio' follicle groups +4 days Most primary X and primary Y fibres have emerged. Primary *x* and primary *y* follicles (tertiary follicles of Gibbs, first underfur follicles of Dry) appear on either side of these to form groups of three follicles. The epidermis is decreasing in thickness and the derma-plus-hypodermis increasing (Gibbs). Connective tissue fibres in the derma form compartments around the trio groups. The hypodermis is now full of fat.

Stage 10 Development of secondary follicles +5 to +8 days Secondary follicles (quaternary follicles of Gibbs, further underfur follicles of Dry) are formed between the central and lateral members of the trio groups. From this time follicle groups are more difficult to distinguish. An arrector pili muscle becomes attached to each follicle (Gibbs). The epidermis continues to decrease in thickness and the derma-plus-hypodermis to increase (Gibbs).

Stage 11 End of follicle length growth +14 days All follicles cease to grow in length. The thickness of the epidermis remains constant, but that of the derma-plus-hypodermis is now diminishing.

Stage 12 First resting phase About +18 days The hairs have become thin at the base, their growth has ceased and 'hair bulbs' are being formed (the end of growth of G1 of Dry). The derma-plus-hypodermis is decreasing in thickness.

Stage 13 Beginning of second growth cycle About +28 days A new generation of 'companion hairs' (G2 of Dry) begins by proliferation of cells at the base of the resting hair follicles.

Melanin pigment is produced in the skin of mice and other mammals in the branched cells known as melanophores (Rawles, 1948) or pigmented dendritic cells (Billingham, 1948). Their colourless precursors, the melanoblasts, are derived from the neural crest (Rawles, 1940, 1947). They are to be distinguished from phagocytic cells in the derma which ingest but do not synthesize pigment granules, and which have not been found in normal mouse skin (Steiner-Wourlich, 1925).

In the grey house-mouse Steiner-Wourlich (1925) found melanophores first in

the derma of an 18 mm embryo (i.e. 17–18 days' gestation, according to the data of Gruneberg, 1943). These pigmented cells became darker and more numerous after birth, and similar cells appeared in the basal part of the epidermis. Soon after birth the hair matrix contained melanophores. Hentschel (1930) found that as the hair grew, melanin granules appeared first in the cortex and then in the medulla.

In the C57 Black strain Rawles (1947) found melanophores were present at birth in the hair matrix and were lightly scattered in the derma. They were absent from the epidermis, except in sparsely haired body regions such as the ears and tail, where they were numerous in both the derma and the stratum germinativum.

THE DEVELOPMENT OF SKIN AND HAIR FOLLICLES *IN VITRO*

Skin pieces were cultivated for periods of from 1–3 weeks and most stages of differentiation of the epidermis, derma-plus-hypodermis and hair follicles were produced. The main results are summarized in Table 1, which shows the most advanced stage of differentiation reached in each experiment. In this section a summary of the results and of the effect of modifications of technique will be followed by a more detailed account of development *in vitro*.

(1) *The numbers of successful cultures*

The degree of success of the tissue-culture method may be judged to some extent from Table 1, but this refers only to the most advanced cultures in each experiment. Table 2 shows the number and percentage of cultures in which some new stages of follicle differentiation produced *in vitro* were recognized in histological sections. The percentages represent minimum values, since many cultures were fixed or discarded in the first few days, and in others progress was sometimes followed by retrogressive changes before fixation. At least 48% of all cultures were successful when all ages, body regions, techniques and growth media were included. When the incomplete figures for Exp. 9 are omitted the percentage is 54. Of the cultures from 12- to 14-day embryos, excluding Exp. 9, 68% were successful.

A further idea of the progress of cultures may be gained from the following details of Exp. 6, in which the percentage of cultures showing some differentiation was close to the average for the 12- to 14-day group.

- 26 cultures were prepared,
- 9 explants developed to Stage 6 (hair fibres),
- 6 explants developed to Stage 4 (fibre cones),
- 1 explant developed to Stage 3 (papillae),
- 2 explants developed to Stage 1 (follicle plugs),
- 7 explants grew without differentiation of hair follicles,
- 1 explant showed no outgrowth.

Two of the six cultures which developed to Stage 4 were fixed at 21 days, and might have produced hairs if cultivated for a longer period.

(2) *The effects of different conditions of cultivation*

(i) *Age of embryos* From Table 1 it may be seen that the most advanced differentiation was obtained from embryos between 12 and 14 days old, but that younger and older tissues also differentiated to some extent. Table 2 shows that the percentage

of successful cultures was highest in the 12- to 14-day series of explants. The slight differences found sometimes between litter-mates in the stage of development were not large enough to affect the course of the experiments.

(ii) *Body regions* Successful cultures were made with dorsal, lateral and ventral parts of the trunk. The mid-dorsal skin of 10- and 12-day embryos was not very suitable for explantation because the ectoderm was particularly thin and fragile,

Table 1 *Summary of tissue-culture experiments*

Exp no	Method	Age of embryo (days)	Initial stage	Final stage <i>in vitro</i>	Minimum duration of culture period for final stage reached (days)	Total age of explant at final stage (days)	Normal age <i>in vivo</i> for the stage reached <i>in vitro</i> (days)
11	Watch glass	10	0	3	<19	< +8	16
12	Watch glass	12	0	8	<18	< +9	+2 to +3
6	Hanging drop	12	0	8	13	+4	+2 to +3
9	Hanging drop	12	0	3	4	16	16
3	Hanging drop	12.5	0	5	8	20.5	20
10	Watch glass	13	0	8	12	+4	+2 to +3
8	Hanging drop	14	0	5	9	+2	20
5	Hanging drop	14	1	8	10	+3	+2 to +3
1	Hanging drop	15	2	3-4	6	21	18
7	Hanging drop	15	2	6	7	+1	21
2	Hanging drop	18	3	6	3	21	21
4	Hanging drop	18	4	6	4	+1	21

Table 2 *Numbers of cultures which showed progress in hair follicle development*

Exp no	Age of embryo (days)	Total no of cultures	No of cultures showing further follicle differentiation	Successful cultures (%)
11	10	6	2	33
12	12	12	7	58
6	12	26	18	69
9	12	38	(8+)*	(21+)
3	12.5	10	8	80
10	13	11	11	100
8	14	36	18	50
5	14	14	12	86
1	15	20	4	20
7	15	36	15	42
2	18	14	6	43
4	18	10	4	40
		Grand total 233	113	48

* Not all the cultures were examined histologically to determine follicle differentiation

and there was little underlying mesoderm. In normal development there are slight differences between regions in the rate of follicle differentiation, but equally great differences were found *in vitro* between explants from the same body region.

(iii) *Hanging-drop and watch-glass cultures* It will be seen from Tables 1 and 2 that the two methods of cultivation were equally successful in producing follicle differentiation. The hanging-drop cultures contained only epidermis and mesoderm, so that the extra tissues such as cartilage included in watch-glass explants were evidently not essential for follicle development. In hanging-drop cultures the explants survived but did not show much progress after about 16 days *in vitro*, while explants in watch-glasses still showed growth of epidermis, derma and follicles after 25 days.

The longevity of the latter may be due to the greater original volume of the explants or to the provision of additional mesodermal tissue and the minimizing of its loss by migration. The introduction of other tissues sometimes resulted in a depression of hair-follicle development. Thus in some watch-glass cultures, when mammary gland rudiments were explanted with the ventral body wall, they grew vigorously at the expense of the follicles. This depression might be due to competition for nutrients, an accumulation of metabolites or a specific inhibitory effect. In Exp. 11 in which a variety of tissues was included in each explant from a 10 day embryo, the gut in ventral halves and the otic capsule in dorsal halves grew and differentiated very rapidly, but hair-follicle development was slow and limited to a few stages. The chief advantages of watch-glass cultures, then, were the provision of extra tissue for mechanical support, and the longevity of explants. The chief advantage of the hanging-drop method was the clear view of the living follicles.

(iv) *The position of explants in hanging-drop cultures* In Exps. 1, 2 and 6 comparisons were made between two methods of placing explants in hanging-drop cultures. In the usual 'on plasma' method the explant was placed on the surface of the medium with the derma next to the medium. In the 'in plasma' method the explant was embedded in the clot with the epidermis next to the cover-slip. In both cases the epidermis tended to roll up into a ball surrounded by derma, and there was no appreciable difference between the degree of differentiation of the cultures.

(v) *Size of explants* In hanging-drop cultures the larger pieces of skin with sides of between 1 and 2 mm. were likely to produce more differentiation than smaller ones.

(vi) *Incubation temperatures* The effects on growth of temperatures of 34 and 37°C were compared in Exps. 1, 2 and 6. The general health of the cultures was very similar, and there was no appreciable difference between the rates of hair-follicle development.

(vii) *Frequency of subcultivation* In both hanging drop and watch-glass cultures the explants were transferred to fresh medium at intervals varying from 3 to 6 days. Intervals of 3 or 4 days were usually not too great for the health of the tissues, and older cultures could be left for 5 or 6 days. Explants incubated at 34°C did not need subculture quite so often as those incubated at 37°C.

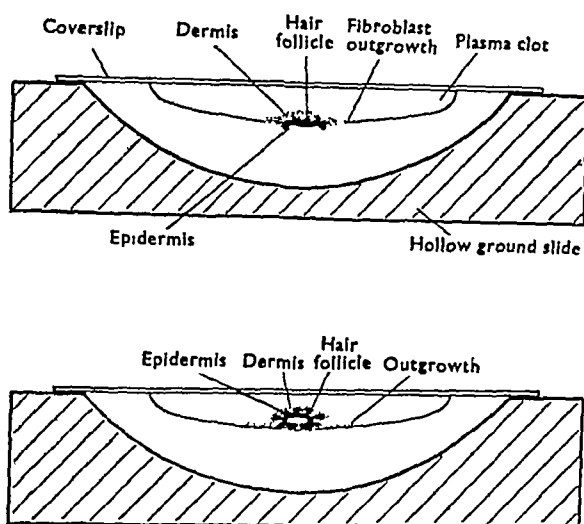
(viii) *Saline component of the medium* In the first ten experiments Tyrode solution was used for washing explants and for preparing the chicken-embryo extract. In Exps. 11 and 12 a comparison was made between Tyrode and Pannett and Compton saline, which lacks glucose and has a different salt balance. No differences were observed between the two groups of explants.

(ix) *Duration of experiments* Each experiment had a slightly different purpose and was therefore continued for a different period. Hanging-drop cultures were maintained for from 7 to 17 days, and usually ceased to grow after about 16 days. Some of the watch-glass explants were still growing when fixed after from 18 to 25 days *in vitro*.

(3) *The general appearance and orientation of explants*

Undifferentiated cells migrated into the medium from all healthy explants. The outgrowth was more extensive in hanging-drop than in watch-glass cultures. Fibroblasts predominated, but epithelial cells were also found in the outgrowth from the skin of older embryos and from all explants after two or three passages.

Skin pieces from 12- to 14-day embryos, which were spread flat on the surface of a plasma clot in hanging-drop cultures, nearly always curled up at the edges on the epidermal side (Text-fig 2). This was associated with a contraction of the ectoderm and with the greater rate of proliferation of mesoderm around and beneath it. After a few days the epidermis frequently rolled into a hollow sphere surrounded by mesoderm. Hair-follicle plugs extended into the mesoderm in directions roughly normal to the dermal-epidermal junction, so that in a flat explant they were more or less parallel to one another but in a spherical one they radiated from the central epidermis in all directions. In the latter case the tips of the hair fibres then grew towards the



Text fig 2 The orientation of skin explants in hanging drop cultures. Cross sections through the centre of culture chambers are represented. In the upper diagram the skin is on the surface of the clot and the epidermis is beginning to curl at the edges. In the lower diagram the epidermis of an older culture has rolled into a ball.

centre of the explant. A great practical advantage of this orientation was that the development of follicles could be clearly seen around the edges of the explants without vision being obscured by the thickening epidermis.

In most of the hanging-drop cultures from 15- or 18-day embryos the explants curled in the opposite direction, so that the epidermis surrounded the derma. The hair follicles were then imprisoned in a sphere of keratinizing epithelium, and showed the effect of isolation from the nutrient medium by their small size and delayed development.

In the watch-glass cultures, in which the explants were many times larger than in hanging-drop preparations, and which contained cartilage and a greater quantity of mesoderm, the epidermis again tended to roll into a ball surrounded by mesoderm. This feature was noted also in rat and mouse embryonic skin in tissue cultures by Hanson (1949), in mouse skin on the chick chorio-allantois by Reed & Alley (1939) and in mouse skin on the chick coelom by Rawles (1940, 1947).

(4) The histogenesis of the epidermis

Hanson (1949) studied the differentiation *in vitro* of epidermis from 12- and 16-day rat embryos and 16-day mouse embryos. From the earliest stage, a single layer of cells in the case of the 12-day rats, a fully differentiated epidermis of stratum germinativum, stratum spinosum, stratum granulosum and stratum corneum was produced in culture. This differed from normal epidermal histogenesis only in the persistence of the periderm and the formation of a hollow cyst by curling of the epidermis. The present observations fully confirmed those of Hanson on the 16-day mouse embryo, and extended the investigation to 10- to 15-day explants. The results may be seen by examining Figs 1-10, which show that the rate of differentiation was comparable with that *in vivo*. The periderm and all layers of the stratum corneum persisted in culture and eventually caused distension of the epidermal cyst (Pl 2, fig 11). Where the stratum corneum was on the outside of the culture and in contact with the plasma, it was frequently abnormal in appearance.

(5) The partial histogenesis of the derma

The mesodermal cells of explants made at Stage 0 multiplied rapidly and many migrated outwards as fibroblasts (Pl 1, figs 3, 5). In hanging-drop cultures much presumptive dermal tissue was lost by outward migration, and even in watch-glass cultures where this tendency was minimized, the derma of old cultures was thinner than in the young mouse. By 21 days, as in normal development, a reticulum of connective tissue fibres had formed in the derma (Pl 1, fig 8, Pl 2, fig 10). These fibres were predominantly parallel to the dermal-epidermal junction. There was no suggestion of a regular fatty layer corresponding to the hypodermis of normal development.

Smooth muscle fibres were sometimes seen, but these were not regularly attached to hair follicles as arrector pili muscles. When parts of the panniculus carnosus were included in cultures, they sometimes persisted.

The immature blood cells in vessels of the mesoderm survived for some days but eventually degenerated. Nerve fibres were not seen in cultures, and presumably they, too, degenerated.

(6) The histogenesis of hair follicles

The first eight stages of hair-follicle development described above, from the appearance of epidermal plugs to the emergence of hairs from the skin, were produced *in vitro*, thus the complete histogenesis of an individual hair follicle may be accomplished in a single tissue culture. Even skin from 12-day embryos, 2 days before normal follicle development (Pl 1, fig 1), was capable of producing hairs in culture, skin from 13- and 14-day embryos was equally successful, and follicle development began in cultures from 10-day embryos (Table 1). Stages 9-13, which were not reported *in vitro*, refer only to the cessation of new follicles and to the cessation of hair growth.

The general shape of the hair follicles and the detailed structure of the dermal papilla, root sheaths and growing hair were strikingly similar to those in normal embryos and young mice. This is best seen by a study of Figs 2-10.

There was much variation between individual explants, but those with healthy growing derma and epidermis usually had a number of normal hair follicles. In general, hair-follicle development *in vitro* in the most successful cultures corresponded exactly to that *in vivo* up to the stage of the 2-day old mouse in which the tips of the hairs are emerging (Stage 8). After that, however, there was little further change in the explants, although the formation of hair from the existing follicles and of stratum corneum continued for some time. Instead of becoming thick, the hairs remained very fine and did not develop a regular medulla, their growth in length was less rapid than in the young mouse, and there were no well-defined clusters of new follicles around the older ones. The longest follicles, lacking the support of a fatty hypodermis, usually lost their radial arrangement and tended to lie parallel to the skin surface (Pl 2, fig 11). Sometimes the epidermal plugs of new follicles which developed later than the reticular fibres of the derma seemed unable to push through this layer, and for that reason they too remained near the epidermis. The sebaceous glands arising from epidermal swellings at the side of the follicles (Pl 1, fig 8) sometimes produced a few differentiated sebaceous cells (Pl 3, fig 18), but did not enlarge further.

(7) *The rate of hair-follicle development*

In Table 1 a comparison is made between the time taken *in vitro* to reach the most advanced stage of follicle differentiation in each experiment, and the time taken *in vivo* to reach this stage on the lateral trunk region. In some cases the development times recorded for cultures were too long because the explants were only fixed at intervals of 3 or 4 days. However, the table indicates that in most experiments differentiation in the best cultures was about as rapid as it is in normal development. The conclusion was the same when intermediate stages were examined. For example, Stage 5 (20 days *in vivo*) was reached by cultures at 20 days in Exp 12 (8 days *in vitro* from 12-day explants) and at 21 days in Exp 10 (13 days *in vivo* and 8 days *in vitro*). Stage 6 (21 days *in vivo*) was reached at 21 days in Exp 6 (12 days *in vivo* and 9 days *in vitro*) (Pl 1, fig 8) and at +1 day in Exp 5 (14 days *in vivo* and 8 days *in vitro*). Figs 2-10 illustrate the similarity of rates of development *in vivo* and *in vitro*.

In hanging-drop cultures it was often possible to observe daily the stage of follicle development, checking the final stage by histological examination. The rates of development of a few typical cultures are compared with the normal rate in Table 3. It will be seen that follicle development in cultures was at about the same rate as in the embryo, although sometimes a day or two faster or slower. On the whole, agreement was remarkably good. There were, of course, some less healthy cultures in which development was slower, and some in which differentiation was followed by retrogressive changes.

(8) *Mitotic activity*

There is a high mitotic rate in the normal skin of embryos and young mice. Hanson (1947) found mitoses in from 2 to 4% of all nucleated epidermal cells between 10 and +10 days. These occurred only in the stratum germinativum and for a short period in the periderm and stratum spinosum.

Qualitative examination of the present material showed that the mitotic rate is high in the stratum germinativum between follicles and still higher in those parts which are forming follicle plugs. Cells of the outer root sheath continue to divide for at least 10 days after birth, while the follicles are increasing in length. Those cells which form the hair matrix divide actively during the period of hair growth. Mitoses are also frequent in the derma for some time after birth.

Table 3 Stages of follicle development in some living cultures

Age in days	Stage <i>in vivo</i>	Exp 5 (age of embryo, 14 days)		Exp 6 (age of embryo, 12 days)			
		No 61	No 63	No 105	No 108	No 112	No 115
13	0	—	—	0	1	1	0
14	1	1	1	0	1	1	0
15	2	1	1	1	—	1	1
16	3	2	2	1-3	3	2	—
17	3	3	2-3	3	3	2	3
18	3	3	3	3	3	3	3
19	4	3-6	3-4	4	4	4	4
20	5	3-6	4	4	4-6	4-6	4-6
21	6	6	6	4-6	6	6*	4-6
+1	7	7	7	4-6	6-8	—	6
+2	8	8*	7-8	6	6-8	—	6
+3	8	—	8	6	6-8	—	6
+4	9	—	8	6*	8*	—	6*

* From histological examination

In general, healthy explants resembled normal skin in the distribution and frequency of mitoses during follicle development. Mitoses were present for a fairly long time *in vitro*. Even after 25 days' cultivation (no 246 in Exp 10), dividing cells were still found in the epidermis, derma and hair follicles.

(9) The size of follicles and hairs

In the embryonic period the healthiest explants developed follicles not only at the same rate as *in vivo* but also of the same size. In the early stages the follicles were sometimes even larger than their counterparts in the embryo, as may be seen by comparing Fig 3 with Fig 2 at twice the magnification. In cultures in which the general health was poor, the follicles were often abnormally small. This was well illustrated in a few explants such as no 249 in Exp 10, in which follicles at Stage 3 situated near the culture medium were very large, while others at the same stage but separated from the medium by a fold of epidermis were abnormally small.

Just after birth, the fully keratinized hairs in the largest follicles of normal skin are from 3 to 6 μ in diameter, and the diameter of follicles at the level of keratinized hairs is about ten times greater. Some follicles are about six times longer than they are wide. The fibres are circular in cross-section and consist of a cortex surrounded by annulate cuticular scales, as described by Dry (1926). Many follicles and fibres of similar shape and size were produced *in vitro* (Pl 2, figs 11-15).

In the first fortnight after birth the follicles in normal skin double or treble their length and the hairs become much thicker, although there is great variation between follicle types, body regions, individuals and strains in these characters. Primary X

and Y fibres may become as much as 30μ wide for a short distance, and diameters of from 10 to 14μ are frequent in all fibre types. Nearly all fibres develop a septate or septulate medulla (Dry, 1926). The medulla occupies half or more of the width of the fibres, so that the increase in volume of keratinized cortex is not as great as fibre thickness measurements would suggest. Some hairs reach a length of 6 mm or more. At about +9 days primary Y follicles and fibres become kidney-shaped in cross-section (Gibbs, 1941).

By contrast, the hairs and follicles in cultures remained small. Follicles sometimes elongated after Stage 8, but not to the same extent as in normal skin. Fibre thickness reached from 6μ (Pl 2, fig 14) to 11μ (Pl 2, fig 11), but rarely more. Occasionally there were traces of medullation, but no regularly septate medullae were formed. Most fibres were round or oval in cross-section and none were kidney-shaped. Growth in length was much slower and no fibres longer than 0.5–1.0 mm were found.

(10) *Follicle types and follicle population density*

The hair follicles which appeared first in culture may be identified with the primary X follicles of the embryo. In normal development the earliest of these follicles are widely spaced at approximately equal distances from one another, and new follicles then appear in the spaces between them (Pl 1, fig 4). The same tendency was observed in some cultures (Pl 1, fig 5). At Stage 7 *in vivo* the primary Y follicles appear, forming irregular rows with the primary X follicles (Gibbs, 1941). In cultures new follicles appeared at about the same stage, but they were not arranged in rows. The later distribution of follicles in groups which is characteristic of mammalian skin and which is definite, though transitory, in the mouse (Gibbs, 1941), was not observed *in vitro*.

Normal follicle grouping and density could hardly be expected in cultures where the skin was no longer stretched over an expanding body surface but was curled up in a ball. No counts were made of the number of follicles per unit of skin area, but in the younger cultures at least the density was roughly comparable with that of corresponding stages *in vivo* (Pl 1, figs 2–8).

(11) *The fine structure of hairs*

Each hair grown in tissue culture consisted of a cortex surrounded by cuticular scales (Pl 2, figs 11–15). Both layers were formed from epidermal cells of the hair matrix by cytological changes similar to those observed in normal skin. Hair formation in culture was preceded by the appearance of an outer root sheath, Henle's layer, Huxley's layer and the cuticle of the inner root sheath in exactly the same manner as *in vivo* (Pl 2, fig 13). The 'keratinized' fibre in the upper part of the follicle and above the skin surface stained differentially in saturated aqueous picric acid in the same manner as the 'keratinized' part of normal hairs, while the hair in deeper parts of the follicle remained unstained with picric acid both *in vivo* and *in vitro*. The physico-chemical basis for the very specific retention of picric acid by the hairs when it is lost from the stratum corneum, root sheaths, medulla and all other skin structures is unknown, but it is a useful distinguishing feature of hair in normal histology. This 'keratinized' portion of hairs fails to take up a wide variety of histological stains in both normal and cultivated skin.

The birefringence of mouse hair follicles was similar to that of human hair which has been described by Schmidt (1934). Freshly plucked mouse hairs mounted in water show an intense positive uniaxial birefringence of the cortex in the direction of the shaft. A weak birefringence is observed a short distance above the papilla, and this increases to maximum intensity near the zone of maximum staining with picric acid. The examination of sections shows that birefringence in the 'keratinized' part of the hair is very much stronger than that of the same thickness of hair in the deeper part of the follicle or than that of the stratum corneum or inner root sheaths. Parts of the inner root sheath are birefringent in the direction of the hair shaft, while the stratum corneum shows orientation in the plane of the skin surface.

In sections of tissue cultures the inner root sheaths, hairs and stratum corneum had normal birefringent properties. The hairs were weakly birefringent in the deeper parts of the follicles, but in the 'keratinized' portion they were very much brighter than any other structures in polarized light. Hairs in living cultures were also strongly birefringent.

The observations described above show that there is a close similarity between the histogenesis of hairs *in vitro* and *in vivo*. Histochemical studies of hair development in the normal animal and in tissue cultures will be reported elsewhere.

(12) Pigmentation

Melanin pigment was produced *in vitro* in the two experiments with watch-glass cultures from pigmented strains of mice. No pigment cells were found in the watch-glass or hanging-drop cultures from the embryos of albino mice.

In Exp. 10, 13-day litter mates of the C57 Black strain were used. Each explant consisted of half of the ventral body wall, with epidermis, mesoderm and developing cartilage. Branched melanophores were found in all of the six explants fixed at +4 days or later (i.e. after 12 or more days *in vitro*), and in one of the two fixed at 21 days (after 8 days *in vitro*). These cells could be distinguished in the living cultures, appearing first in the derma (Pl. 3, fig. 16) or in the derma and hair follicle matrix (Pl. 3, figs. 19, 21) between 21 and +2 days. Pigmented hairs (Pl. 3, figs. 20-22) were first recognized between +6 and +8 days, and sections revealed that melanophores were sometimes present also in the deeper part of the epidermis. In one explant, in addition to melanophores in the stratum germinativum, pigment granules were seen in some cells of the stratum spinosum (Pl. 3, fig. 17).

In Exp. 12 the source of the 12-day embryos was the laboratory stock of mice of agouti colouring. No melanophores were found in any of the five explants of dorsal or ventral body wall fixed at 21 days. They were present, however, in two of the three explants of dorsal body wall fixed at +5 days and in one of two explants of ventral body wall fixed at +9 days. It was seen from the living cultures that melanophores appeared in these explants between 18 and +2 days, and pigmented hairs between +5 and +7 days. The melanophores were first seen in the derma or in both the derma and hair matrix. Sometimes they were recognized early as pale branched cells which became gradually darkened with melanin in the next 1 or 2 days. The histological sections showed melanophores in the epidermis as well as in the derma and hair matrix. Pigment granules were found in the cortex of hairs in two explants, and in one of these some of the differentiating cells of the epidermis also contained

pigment Hair-follicle development was a little slower in these cultures than *in vivo* (Table 1), and when this is taken into account the time of appearance of melanophores in various layers of the skin can be regarded as normal

Another interesting observation was made in certain cultures in each of the above experiments. Some of the hair follicles failed to produce normal keratinized fibres *in vitro*, forming only cords of nucleated cortical cells. Many such follicles had melanophores in the hair matrix, and pigment was produced and extruded as though normal hairs were present to receive it, so that a cylinder of clumped melanin granules was pushed to the skin surface (Pl 3, figs 18, 19). It was not possible to determine whether the pigment cylinders consisted of dead melanophores or merely of granules deposited from them. The process was observed both in living explants and in fixed material. The absence of normal hair formation was confirmed in sections, where there was no normal hair outline, marked periodic acid affinity or strong birefringence in the pigment cylinders. Sometimes normally keratinized and pigmented hairs were found in the same explant as these pigment masses (Pl 3, fig 22). A few irregular pigment masses were found extracellularly in the derma in some old cultures (Pl 3, fig 16), but these were much less conspicuous than the cylinders from hair follicles. When an explant contained many hair follicles the melanophores were concentrated in them, but when hair-follicle formation was deficient, the pigment cells were numerous in the derma and sometimes also in the epidermis. No extensive migration of individual melanophores was observed and none was found in the outgrowth of fibroblasts and epithelial cells.

DISCUSSION

Problems of histogenesis

Nothing is known of the factors which induce hair-follicle formation from the mammalian skin. The cause of the growth of new hair follicles from the base of old ones in the hair cycles of rodents is also unknown, although this is associated with an increased blood supply (Haddow, Elson, Roe, Rudall & Timmis, 1945), and with many other physiological factors. It is interesting to note, therefore, that in the present experiments hair follicles formed *in vitro* in the absence of any tissues except the epidermis and underlying mesoderm. Chemo-differentiation of the skin for hair-follicle formation apparently occurs before the tenth day in the mouse embryo, and the work of Rawles (1947) with coelomic grafts suggests that it may take place as early as the eighth day. Tissue cultures have shown also that neither a blood circulation nor nervous stimulation is necessary for the initiation of follicles, although both blood vessels and nerves were present in the mesoderm at the time of explantation. Any influence of fluids normally bathing the surface of the embryo is also excluded. A factor stimulating the formation of hair follicles is extremely unlikely to be present in a culture medium of avian origin, especially as the time of follicle initiation depended on the total age of the tissues and not on the duration of cultivation. It seems most probable that the embryonic ectoderm and mesoderm are not dependent on any outside influences for follicle formation.

The pattern of hairs in the skin presents many interesting problems. As in other mammals, the first, or primary *X* follicles in the mouse are rather regularly spaced,

and later follicles appear in positions related to them. Either this pattern is already laid down in the skin or each developing follicle becomes surrounded by a small zone where development is inhibited. There are difficulties in accepting either theory. The present studies have thrown little light on this problem except to show that an equally regular spacing of primary X follicles can be obtained in skin removed from blood capillaries and nerves.

Not only could the differentiation of hair follicles begin *in vitro*, but it could proceed to completion with all the many morphologically distinct layers in normal relation to each other. This complete self-differentiation took place in a relatively constant medium, entirely avian in origin. The follicle parts were formed in the right order and at the normal rate, and they were the same size as those *in vivo* during the differentiation period. Thus the medium, at least qualitatively, lacked none of the essential requirements for follicle differentiation and hair growth from ectoderm and mesoderm.

In the fowl it was shown by Lillie & Wang (1944) that the dermal component of a feather papilla induces differentiation in the epidermal component. It is possible that a similar state of affairs exists in the hair follicle, this has not been investigated. In tissue cultures it was noted that no epidermal plugs formed in the absence of mesoderm, and that dermal papillae could not be distinguished in areas from which the epidermis had been removed.

Hair canal formation

Gibbs (1938) described the events leading to the formation of a hair canal in the epidermis above a developing follicle in *Trichosurus vulpecula*. A plug of cells connected with the differentiated sebaceous gland pushes outwards, causing the cells of the stratum spinosum in front of it to disintegrate, forming a small canal which is quickly keratinized. A similar process was reported in the sheep (Wildman, 1932) and the mouse (Gibbs, 1941), but not in mammals generally, where the formation of the hair canal is usually regarded as a function merely of the upper layers of the epidermis.

In the present study, a connexion between the sebaceous gland and the formation of a hair canal was not satisfactorily established in normal mouse skin.

Hair canals in the mouse were observed *in vitro* in hanging-drop cultures, both in the living tissue and in sections. Although in explants a sebaceous gland swelling usually appeared at the time of hair canal formation, no differentiated sebaceous cells were seen at this period. The few follicles which produced differentiated sebaceous cells did so at a much later stage. The hair canals were formed by the keratinization of cells in the epidermis, and appeared to be quite normal. No connexion was observed between the sebaceous gland swelling and the hair canal and it seems that in the mouse skin, at least *in vitro*, differentiation of the gland cells is not essential for the development of a hair canal.

Pigmentation

Rawles (1940, 1947), using grafts to the chick coelom, showed that the precursors of pigment cells had migrated to all parts of the skin of the mouse by the twelfth day of gestation. The present observations on tissues cultivated in a non-cellular

medium confirm this result. Unlike those of dominant and recessive white breeds of fowls (Hamilton, 1940) tissues from the albino mouse did not produce melanophores *in vitro*. However, it is possible that white dendritic cells are present, as in spotted guinea-pigs and albino rabbits (Billingham, 1948) or that the melanophores are less viable than in the pigmented strains, as is the case with fowls (Hamilton, 1940).

The differentiation *in vitro* of mammalian melanophores is apparently very similar to the process in tissue cultures from birds (Koller, 1929, Dorris, 1938, Hamilton, 1940). To the knowledge of the present writer the only previous observation of mammalian pigment cell differentiation in culture was that of Murray (1933*a*). The deposition of pigment granules from melanophores into the medium was observed in chick explants by both Dorris and Hamilton, while Grand & Cameron (1948) described the same process in cultures of mouse melanoma tissue. It has now been demonstrated that melanophores can also deposit their pigment in the hair cortex *in vitro*. The process was similar to that in the normal mouse as described by Steiner-Wourlsch (1925). The few differentiating epidermal cells which contained pigment were first seen after the epidermal melanophores had appeared and were only found in areas where the latter were numerous. It therefore seems likely that melanin in these epidermal cells also was derived from melanophores.

No extensive migration of dendritic cells was observed after they had become pigmented, and in this respect they resembled the fowl melanophores *in vitro* (Dorris, 1938, Hamilton, 1940).

No evidence was found in support of the belief of Steiner-Wourlsch (1925) and Hentschel (1930) that the epidermal and hair-matrix melanophores are independent of the dermal melanophores. On the contrary, observations *in vitro* support the suggestion of Rawles (1947) that there is a balanced distribution of melanophores between the three regions. No morphological difference was observed *in vitro* between pigment cells of the derma and epidermis.

Although the pigmentation process in normal skin and hair is easily upset by changes in the environment (Reed & Henderson, 1940, Butcher, 1945), melanophores of the mouse skin in tissue culture were much less sensitive than those of pigmented breeds of fowl (Hamilton, 1940). Pigmentation could even occur normally *in vitro* in hair follicles in which normal keratinization was not taking place. The tissue-culture technique may, therefore, be useful for studying some of the outstanding problems of melanin formation in mammals.

Comparison of the tissue-culture technique for studying hair growth with some other experimental methods

The present experiments on embryonic mouse skin were much more successful in producing the differentiation *in vitro* of the follicles of pelage hairs than were those of Strangeways (1931) on the guinea-pig or Murray (1933*b*) on the rat. Strangeways cultivated the skin of embryos of various ages but apparently did not obtain hair-follicle differentiation of more than one or two stages. Both she and Murray (1933*b*) referred to the thick stratified epidermis which interfered with the normal development of the follicles of pelage hairs. It may be that the thinner epidermis of the mouse is more suitable for *in vitro* studies of hair follicles.

Varying degrees of success in hair-follicle development were reported by those using the chick chorio-allantoic graft method (Hiraiwa, 1927, Nicholas & Rudnick, 1933, Reed & Alley, 1939). Even the most successful and highly vascularized grafts of Reed & Alley did not persist as long or produce follicle differentiation as consistently as the tissue cultures.

Grafts of the embryonic skin of the mouse to the chick coelom were much more satisfactory. In the first experiments by Rawles (1940) 61% of grafts 'took', and in a later series (Rawles, 1947) 77% were successful. Hair follicles and hairs were produced in most of these implants. Rawles noted that hair development was more abundant from 11- to 12-day embryonic grafts than from 8- to 10-day tissues. An illustration to the second paper shows that one graft at least had medullated hairs from 7 to 12 μ thick and about 700 μ long. At a total age of +10 days, some hairs were said to be several millimetres long. The hair follicles were normal in histological detail and very similar to those grown in tissue cultures in the present experiments. Tissue cultures and coelomic grafts thus illustrate to a similar degree the capacity of mouse skin and hair follicles to differentiate in an avian environment. A greater volume of keratinized hair was produced by the coelomic graft method, probably because of the presence of a blood circulation.

It is considered that the tissue-culture techniques described in this paper possess some advantages over all of the grafting techniques for the purpose of studying the effects of the non-cellular environment. The nutritive medium, though complex, is relatively constant, and its chemical constituents may be analysed at any time during the experiment. Any nutritive substance, enzyme or inhibitor may be added to the medium to produce its effect directly on the skin. Tissue culture permits the continuous observation of histological changes in hair follicles in a way that is not possible by any other technique.

Limiting factors to continued hair growth

There are many possible reasons why hair growth cannot at present be maintained indefinitely. Since differentiation and the synthesis of hair keratin is completed in culture, nutritional deficiencies are likely to be quantitative rather than qualitative. There may be too little of the raw material or of the enzymes for hair synthesis, or these may be in the wrong proportions. It is interesting to note that there was no apparent reduction in the rate of formation of keratinized stratum corneum. The stimulus to cell division may become inadequate, yet a high mitotic rate is maintained in the epidermis. Mechanical factors arising from the abnormal orientation of follicles may be partly responsible, but they did not prevent good hair growth in the chick coelom grafts of Rawles. The better results from coelom grafts suggest the importance of vascularization.

Some of these possibilities will be investigated in future work.

SUMMARY

1 Skin from the trunk of mouse embryos of 10-18 days' gestation was grown in hanging drop and watch-glass cultures on a mixture of fowl plasma and chicken-embryo extract.

- 2 Some differentiation of hair follicles took place in embryonic skin of all ages, but the best results were obtained from the skin of 12- to 14-day embryos
- 3 All stages of hair-follicle development up to the emergence of hairs from the skin were produced from undifferentiated ectoderm with some underlying mesoderm
- 4 The histological structure of the hair follicles and hairs formed in culture was normal
- 5 In the most successful cultures, follicle differentiation took place as rapidly as in the embryo, and the follicles and hairs were of normal size. When the tissues reached an age corresponding to the second day after birth, hairs continued to grow for about a week but they did not become as large as in the young mouse
- 6 Dendritic cells appeared in culture, developed melanin granules and deposited these in growing hairs *in vitro*

This work was undertaken during the tenure of an Overseas Studentship of the Council for Scientific and Industrial Research (Australia). I am grateful to Dr H B Fell for providing facilities at the Strangeways Research Laboratory and Prof J Gray for facilities at the Department of Zoology, Cambridge. Dr Fell's guidance in tissue-culture methods and her interest and encouragement are greatly appreciated.

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EXPLANATION OF PLATES

Photographs by Mr V C Norfield and Mr G Lenney

<i>C</i>	Nucleus of hair cortex cell	<i>MG</i>	Melanin granule
<i>Cr</i>	Stratum corneum	<i>Ms</i>	Mesoderm
<i>Cu</i>	Nucleus of hair cuticle cell	<i>O</i>	Outer root sheath
<i>CuI</i>	Nucleus of inner root sheath cuticle	<i>P</i>	Dermal papilla of hair follicle
<i>F</i>	Fibre cone	<i>PC</i>	Panniculus carnosus muscle
<i>G</i>	Stratum germinativum	<i>PP</i>	Pre papilla
<i>Gr</i>	Stratum granulosum	<i>Pr</i>	Penderm
<i>H</i>	Hair	<i>R</i>	Reticular layer of derma
<i>HC</i>	Hair canal	<i>S</i>	Stratum spinosum
<i>He</i>	Henle's layer	<i>SG</i>	Sebaceous gland swelling
<i>Hx</i>	Huxley's layer	<i>X₁</i>	Early primary X follicle
<i>M</i>	Melanophore	<i>X₂</i>	Later primary X follicle

PLATE 1

- Fig 1 Vertical section of skin of 12 day mouse embryo at Stage 0, showing the epidermis and under lying mesoderm with immature blood cells (control to Exp 6) Haematoxylin, chromotrope $\times 572$
- Fig 2 Vertical section of skin of 15 day mouse embryo at Stage 2, with two early follicle plugs and one larger follicle at the pre-papilla stage (control to Exp 7) Haematoxylin, chromotrope $\times 185$
- Fig 3 Horizontal section of an entire culture of skin from a 12.5-day mouse embryo after 3 days *in vitro* (no 37, Exp 3) The explant has reached Stage 2, and several hair follicles are seen in the section Haematoxylin, chromotrope $\times 93$

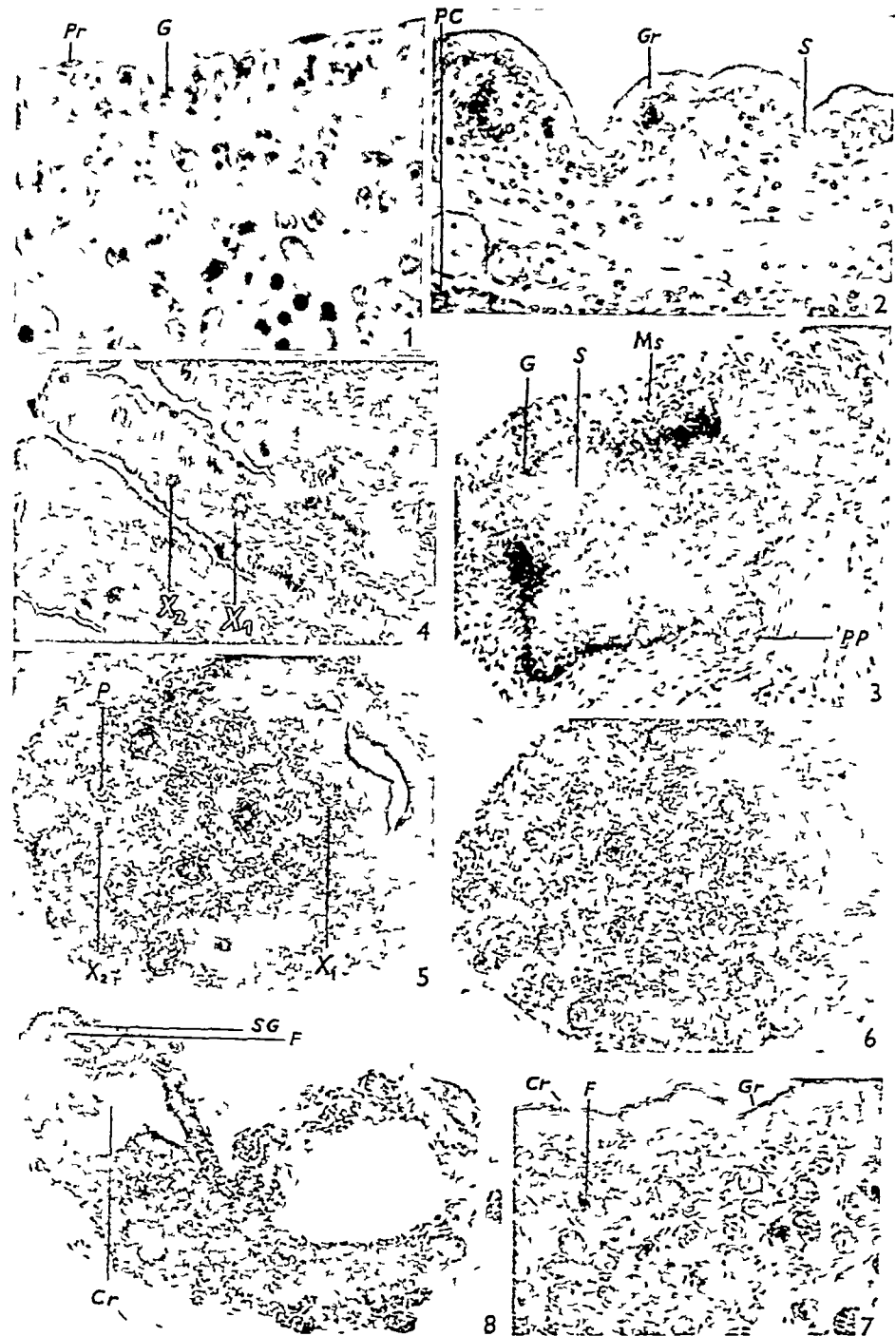
- Fig 4 Horizontal section of skin of a 16 day mouse embryo at Stage 3, showing the pattern of early and late primary X follicles Haematoxylin, chromotrope $\times 59$
- Fig 5 Horizontal section of an entire culture of skin from a 14 day mouse embryo after 3 days *in vitro*, showing follicles at Stage 3 (no 66, Exp 5) Haematoxylin, chromotrope $\times 59$
- Fig 6 A deeper section of the same culture showing the arrangement of the earliest primary X follicles in rows Haematoxylin, chromotrope $\times 59$
- Fig 7 Oblique section of skin of newborn mouse at Stage 6, showing hair follicles of various sizes, the largest containing minute hair tips inside the fibre cones Haematoxylin, chromotrope, picric acid $\times 90$
- Fig 8 Horizontal section of a culture of skin from a 12 day mouse embryo after 9 days *in vitro*, at Stage 6 and showing many hair follicles (no 129, Exp 6) Haematoxylin, chromotrope, picric acid $\times 90$

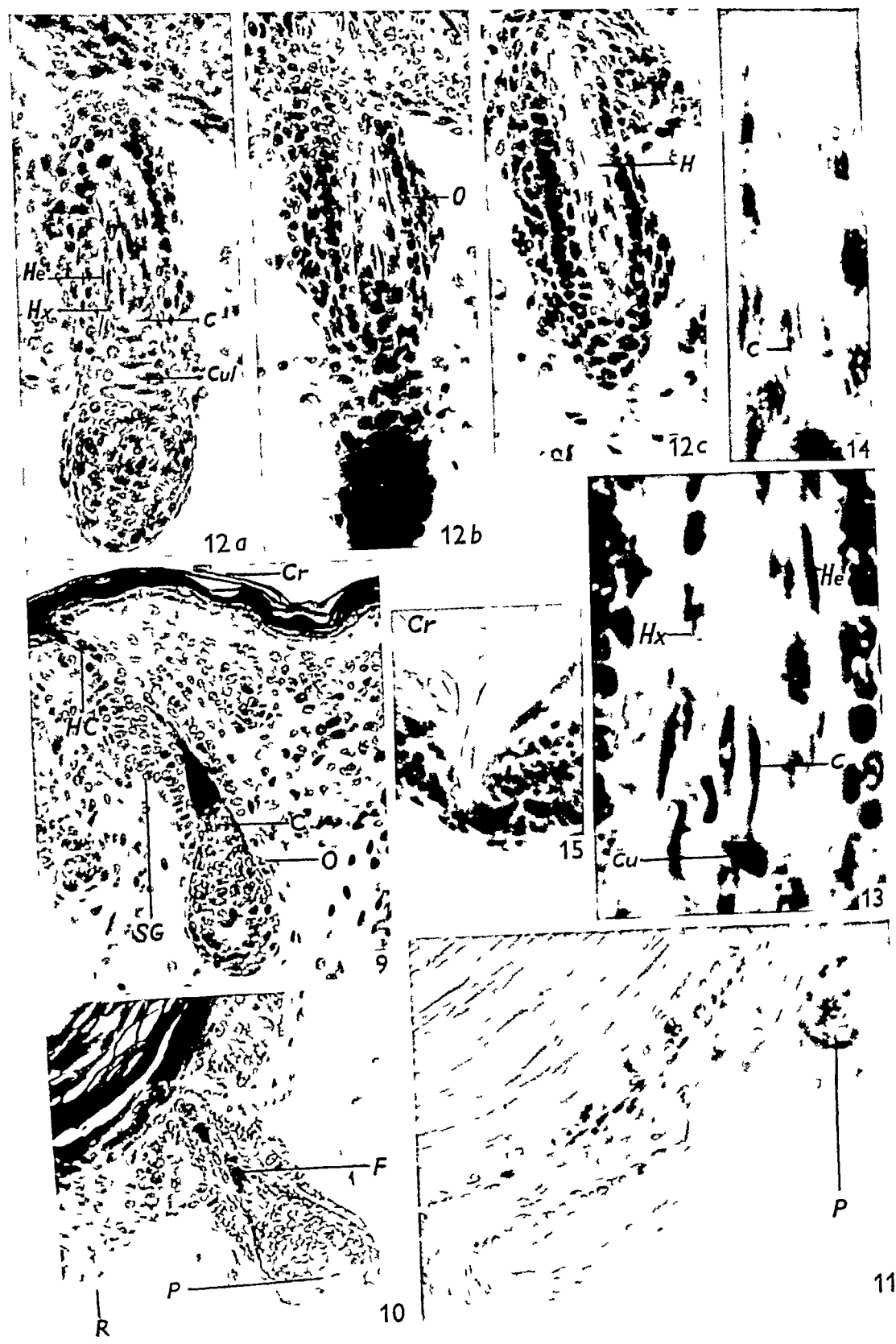
PLATE 2

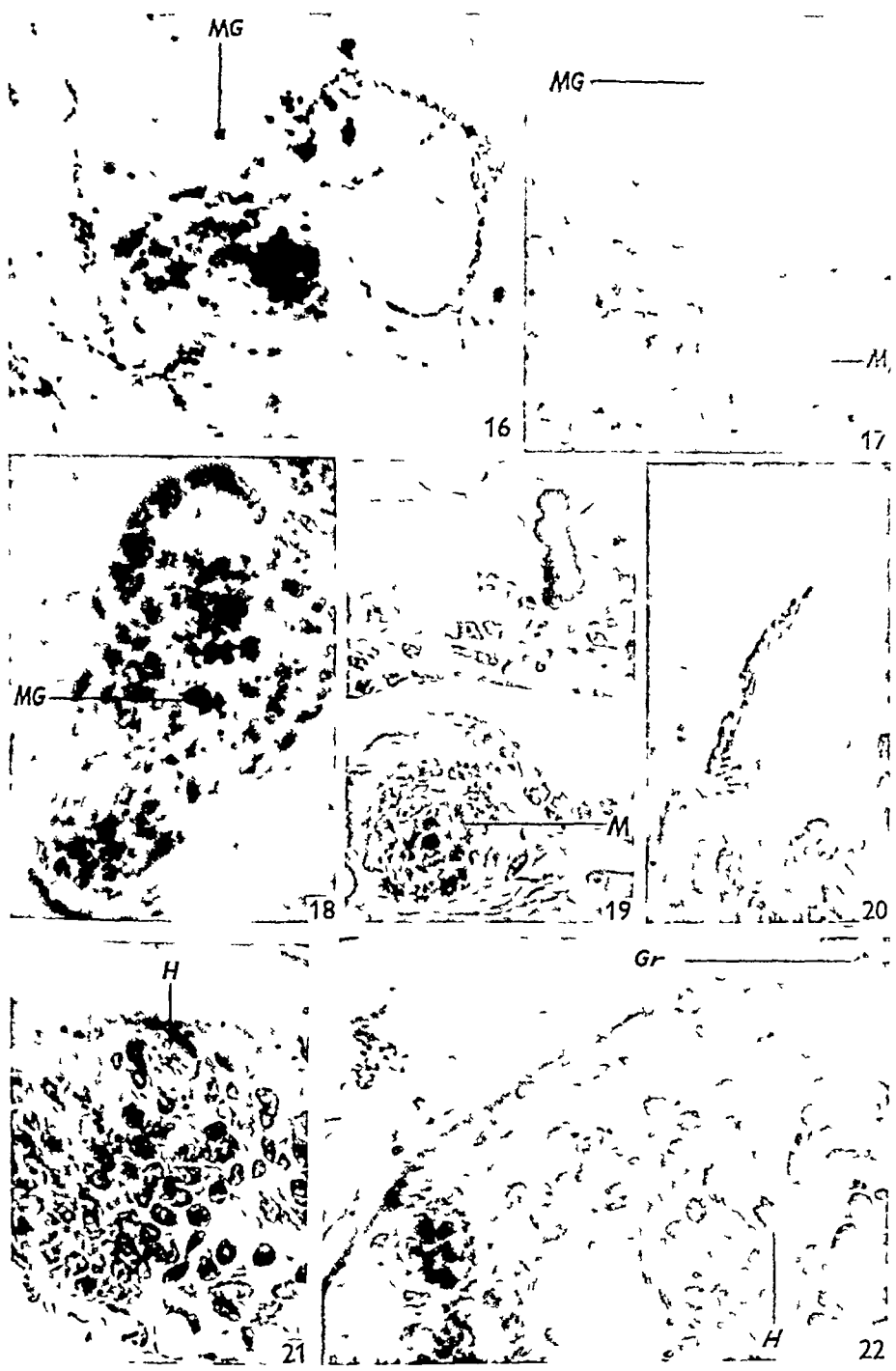
- Fig 9 A hair follicle in the skin of a newborn mouse at Stage 5 The epidermis is fully differentiated, and two younger follicle plugs are also seen Azan $\times 243$
- Fig 10 Part of a culture of skin from a 12.5 day mouse embryo after 8 days *in vitro*, showing a follicle at Stage 5 (no 45, Exp 3) The epidermis is fully differentiated and the centre of the explant is filled with stratum corneum Azan $\times 243$
- Fig 11 Portion of a culture of skin from a 14 day mouse embryo after 16 days *in vitro*, showing two follicles lying in the derma parallel to the skin surface (no 63, Exp 5) Haematoxylin, chromotrope, picric acid $\times 270$
- Fig 12a, b, c Three adjacent sections of a hair follicle in skin from a 13 day mouse embryo after 12 days *in vitro* (no 250, Exp 10) Haematoxylin, chromotrope $\times 369$
- Fig 13 Part of the section shown in fig 12b at higher magnification showing the follicle sheaths and the formation of the hair from the cortex and cuticle cells Haematoxylin, chromotrope $\times 1260$
- Fig 14 The field shown in fig 13 at a different focal depth to show the surface of the hair with its cuticular scale pattern Haematoxylin, chromotrope $\times 1260$
- Fig 15 A hair emerging from its follicle mouth in a culture from a 13 day mouse embryo after 19 days *in vitro* (no 241, Exp 10) Haematoxylin, chromotrope, picric acid $\times 225$

PLATE 3

- Fig 16 Part of a culture from a 13 day mouse embryo after 25 days *in vitro*, showing two melanophores in the derma (no 246, Exp 10) Haematoxylin, chromotrope, picric acid $\times 765$
- Fig 17 Part of the epidermis in the same culture, showing melanophores in the stratum germinativum and processes from these cells between the cells of the stratum spinosum Some epidermal cells in the stratum spinosum and stratum granulosum contain melanin granules Haematoxylin, chromotrope, picric acid $\times 396$
- Fig 18 Section through a hair follicle in the same culture, showing melanin granules in the follicle above the dermal papilla, and a small sebaceous gland Haematoxylin, chromotrope, picric acid $\times 603$
- Fig 19 Part of a culture from a 13 day mouse embryo after 22 days *in vitro*, showing a cylinder of melanin pigment which has been extruded from a follicle (no 247, Exp 10) Haematoxylin, chromotrope, picric acid $\times 599$
- Fig 20 Part of the same culture, showing a normal pigmented hair Haematoxylin, chromotrope, picric acid $\times 855$
- Fig 21 Part of the same culture, showing a normal pigmented hair in transverse section Haematoxylin, chromotrope, picric acid $\times 599$
- Fig 22 Part of the same culture, showing on the right a normal heavily pigmented hair and on the left a follicle which is extruding pigment granules Haematoxylin, chromotrope, picric acid $\times 599$







REVIEWS

Neurological Anatomy in relation to Clinical Medicine By A BRODAL (Pp xv+496, 80 figs, 3 plates 1948 42s) Oxford University Press

The part which anatomy plays in the field of clinical neurology is well recognized but, in view of the tendency to extreme specialization and the mass of literature which daily accumulates, there is obvious need to review the whole field of neuro anatomy and to attempt a correlation with neuro physiology and clinical neurology. This difficult task is ably undertaken by Dr Brodal, who wisely avoids the tendency to produce rigid schemata which so often prove a stumbling block to further progress. Thus he demonstrates not only the extent to which anatomy assists in the understanding of clinical manifestations, but also the points on which academic findings are inadequate, so providing a stimulus to further effort in the anatomical field.

It is implicit in a work of this nature that agreement on all points is not to be expected and that it is impossible for one author to have first-hand knowledge of the whole range of this rapidly expanding subject. Dr Brodal minimizes these difficulties by quoting his sources of information throughout the text and provides an excellent selection of references. These, together with a valuable introductory chapter on the significance of the various neuro-histological techniques, should assist those not directly conversant with the limitations of such procedures to develop a critical approach to the subject.

The book is divided into a series of chapters, each dealing with a separate functional system in terms of its anatomy, physiology and the manifestations of experimental and pathological lesions.

The order in which Dr Brodal presents the various systems is difficult to understand. For example, the introductory chapter is followed by a consideration of the spinal motor neurons. The reader is then plunged into a section dealing with the pyramidal tract and motor area of the cerebral cortex, before reaching the portions concerned with the 'extra-pyramidal' systems and the sensory apparatus. Although it is clear from the text that the author fully appreciates the importance of the 'extra-pyramidal' system in voluntary movements, the above order tends to obscure the close interrelation of pyramidal, extra-pyramidal motor mechanisms and the sensory system in the control of muscular activity. It is interesting that Dr Brodal, in an excellent chapter on the cerebellum, considers first the afferent and then the efferent pathways of this organ with a great increase in clarity compared with the method followed in the book as a whole.

It is perhaps unfortunate that the vascular system with its importance in the origin and development of many pathological processes receives only passing mention. A few minor inaccuracies which have here and there crept into the text might well be altered in later editions. For example, it is stated in relation to the organ of Corti that the apical coil mediates sounds 'of the highest pitch'.

Despite these criticisms and the fact that the clinical descriptions at times outweigh the anatomy, this book should prove of very considerable value to those already acquainted with the gross anatomy of the nervous system. It will appeal to advanced undergraduate and postgraduate students as well as to the clinical neurologist who should find it useful not only for its intrinsic value but also as a source of references for further study.

The book as a whole is pleasantly produced. The printing and reproduction of diagrams and plates is excellent and, despite the fact that much of it is the author's translation of the original publication in the Norwegian language, it is readable and easily comprehended.

G J ROMANES

Cunningham's Manual of Practical Anatomy Revised and edited by JAMES COUPER BRASH, M C, M A, M D, F R C S Ed, F R S E 11th ed vol 1 (General Introduction, Upper Limb, Lower Limb Pp x+387, 191 figs), vol 2 (Thorax and Abdomen Pp x+488, 238 figs), vol 3 (Head and Neck Brain Pp x+513, 230 figs) Cr 8vo Each vol 21s net Oxford University Press Geoffrey Cumberlege

Just 70 years after the publication of Prof Cunningham's *Dissector's Guide* there now appears the 11th edition of his *Manual of Practical Anatomy*. The new edition appears under a single editorship, and in his Preface Prof Brash pays a graceful tribute to his collaborator in the two previous editions, Dr E B Jamieson. Prof Brash is to be congratulated on achieving the rare distinction of bringing out a new edition with fewer instead of more pages than the previous one.

The main change between this and the 10th edition of 1940 is in the reduction of the number of pages and in the quality of the paper, which have combined to form a series of much smaller volumes. The new edition differs from the reprints of the 10th chiefly in the order of dissection of the brain, which now proceeds from the brain-stem upwards. The new, almost 'art', paper employed gives a bright, white surface to the page, but one fears that it may show, even more than usual, the marks of the wet and greasy fingers of the dissectors, but this is a misfortune that must befall any book brought into the dissecting room. It does seem that this paper is apt not to take the type well, and this is found in several of the figures, particularly the radiographs in vol 1. On some pages a curious heavier type suddenly appears among ordinary type.

A number of new radiographs of injected vessels have been introduced into vol 1. Though these show more vessels than the old Plates which they replace, the vessels are almost too numerous and the radiographs are rather dark, so that it is not easy to identify some of the structures.

They are bright, pleasant books to use, neat and handy to slip into the pocket, and the series will surely maintain the high reputation of Cunningham's as a dissecting manual.

C M WLST

Evolution of the Forebrain By G W H SCHEPERS (Pp 207 and bibliography 1948 50s) Cape Town, South Africa Maskew Miller, Ltd

This monograph, which claims to deal with the fundamental anatomy of the telencephalon, is based on the author's study of the structure of the brain of a chelonian reptile, *Testudo geometrica*. About half of it is taken up with a description of the arrangement of nerve cells and fibres and of the blood vascular system in the forebrain of this animal, the rest consists of a very speculative account of the structural and functional principles involved and of the evolution of the forebrain from invertebrate ancestors to man. Considerable use is made of literature earlier than 1935, but there are few references to work later than this date.

The descriptive part of the work is of some value. It is based on the study of serial sections stained by the usual neurological techniques including silver impregnations and Golgi methods. It does not, however, go further than several similar descriptions which have been published for other reptilian brains and there is little evidence for any critical appreciation of the limitations of the methods used. So far as one can tell, since there are few if any references to particular preparations or techniques, the description is based principally on the appearances in a transverse series prepared by a Cajal silver impregnation method, a series which is illustrated by a number of drawings. The other illustrations, which are numerous, are with few exceptions diagrams illustrating the author's morphological and functional interpretations. It is not clear what use, if any, has been made of the Golgi material, and for these reasons among others it is difficult to assess the value of his interpretations. This is particularly unfortunate, since, from his observations in *Testudo geometrica* the author is frequently led to a reinterpretation of the findings of others in

different reptilian brains with which, apparently, he has no first-hand acquaintance. One may also doubt whether some of the morphological features described, such as shallow grooves on the surface of the brain or small branches of blood-vessels, are either definite or constant enough to carry the weight of theoretical interpretation which is placed upon them.

The second part of the work which attempts to deal with the fundamental principles of forebrain morphology ('morphological fundamentalism', to use the author's term) is too obscure and has too little foundation on observation to merit serious consideration. The argument becomes almost wholly abstract and speculative, and divorced from the materials with which neuro-anatomists are accustomed to work. Detailed criticism is unnecessary.

The author introduces a new or modified terminology for the reptilian brain which seems to have few advantages. There is no index, complicated abbreviations and symbols are used not only for labelling the figures, but also in referring to particular structures in the text, and no alphabetical key to these abbreviations is provided. The book is therefore difficult to read and loses most of any value it might have as a descriptive account of a chelonian brain.

F GOLDBY

Lehrbuch der Embryologie By W BRANDT (Pp xii+648, 472 illustrations 1949
Swiss fr 56) Basel S Karger

'Die Entwicklungsgeschichte ist der wahre Lichtträger für Untersuchung über organische Körper' It is with this quotation from von Baer that Dr Brandt ends the text of this admirable new survey of human development. A reading of the text makes it obvious that the author does not pay mere lip service to the von Baer dictum. He uses the embryological torch not only to illumine adult relationships and developmental abnormalities but also to throw light on the wider biological significance of the facts recorded. Further, and as was to be expected in a book by one who has made important contributions to our knowledge of development, much of the text is first hand and the whole of it is permeated by a real awareness of the biological status of the problems involved.

Dr Brandt divides the subject matter of his volume into two parts, one on general and the other on special embryology. The general part occupies the first 171 pages of the book and deals with such topics as the sex cells, fertilization, cleavage, the germ layers, the organizer mechanism and determination. It also includes an account of the early development of the human embryo, of embryonic and foetal growth and of twinning and descriptions of implantation, of placental development and of general reproductive physiology. This section appears, to the reviewer, to be both succinct and well informed. Indeed, apart from detail, it can only be suggested that the description of menstruation should come before that of implantation and that the account of foetal measurements is a little too detailed.

The second part of the book extends over 456 pages and is concerned with organogenesis and with certain related problems of developmental physiology. It includes an excellent summary of the author's micro-manipulative technique for the experimental study of the development of organs. This section of the book does not appear to be quite as satisfactory as the first. This is doubtless due to the extraordinary difficulty, with the present embarrassing richness of our knowledge of descriptive embryology, of presenting organogenesis in a well-balanced manner. Personal knowledge of and, in a sense, special affection for certain organs or regions is bound to result in an over-emphasis of some aspects at the expense of others. Nevertheless, the reader of this section will find much that is new, at least to those who have not followed the German literature of the last twenty years, and he will be stimulated in particular by the descriptions of the determination problem as it affects, for example, the development of the gut and its derivatives and of the joints.

So far as the reviewer can judge the text is well written. At any rate the German is not difficult to follow. The book is well printed and produced and many of the figures are excellent. A great defect, however, is the absence of a bibliography. This absence is the more surprising, and tantalizing, in that there are many author references, with dates, in the text. There is, however, a good subject index.

J D BOYD

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